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IODIDE TRAPPING WITHOUT ORGANIFICATION IN A TRANSPLANTABLE RAT THYROID TUMOR[1]

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ABSTRACT

The present report describes a transplantable rat thyroid tumor that concentrates 40–70% of an injected dose of I^{131}-iodide and loses it rapidly with a half life of 4–8 hours. The I^{131} in the tumor is 98–99% iodide as judged by trichloracetic acid solubility, chromatography and dialysis. The remainder of the I^{131} is organically bound and exists in the "particulate" fraction. Iodide accumulation *in vivo* appeared to be independent of the level of thyrotropic hormone.

Slices of the tumor concentrate iodide with a gradient (T/M ratio) ranging from 8 to 44. The gradient is largely abolished by various anions such as ClO_4^-, $SeCN^-$, SCN^- and by certain agents which uncouple oxidative phosphorylation, such as dinitrophenol and dicumarol. Thyroxine and triiodothyronine have no significant effect on the T/M ratio at $1 \cdot 10^{-4} M$.

The defect in hormone synthesis seems to lie with the iodinating system since unlabeled thyroglobulin appears to be present. The ability to deiodinate L-diiodotyrosine has also been lost.

The iodide concentrating mechanism of the tumor differs from that of normal sheep thyroid tissue by its markedly decreased sensitivity to certain cardiac glycosides which were shown to inhibit thyroidal iodide concentration.

IN 1950 Stanbury and Hedge (1) first reported a family of goitrous cretins whose thyroid glands had lost the ability to synthesize the thyroid hormones but could still accumulate iodide. This iodide could be discharged to a large extent by the administration of SCN^-. Such a defect has since been described by several others (2, 3), and most recently, Morgans and Trotter (4) have reported a partial defect of this type in two siblings in whom nerve deafness was associated with goiter. The two children had apparently synthesized sufficient hormone for normal growth and intelligence, but I^{131} accumulated in their thyroid glands could be partially

[1] Presented at the meeting of the American Goiter Association, San Francisco, California, June 18, 1958.

discharged by the administration of perchlorate. This phenomenon is not demonstrable in the normal thyroid, presumably because trapping of iodide is the rate limiting step in the biosynthesis of thyroxine by normal thyroid tissue.

It has recently been our good fortune to discover a similar defect in a transplantable thyroid tumor of rats and the present report deals with experiments performed in this preparation.

EXPERIMENTAL

The tumor employed in this study was one of a series of transplantable thyroid tumors in the rat developed by Dr. S. H. Wollman of the National Cancer Institute, and designated "Line 1–5A" in his classification (5). The initial tumors were generously supplied by Dr. Wollman and the studies reported here cover generations 15 through 21 of this tumor. They were transplanted subcutaneously into the right and left flanks of female rats of the Fischer strain with 100% "takes," and were allowed to grow for from four to ten weeks. The rats were maintained on Purina Laboratory Chow and water *ad lib.* plus greens once a week. Transplantation into male Sprague-Dawley rats gave no "takes."

For *in vivo* studies, tumors of sufficiently small size were chosen to be seen in their entirety through the one inch hole of the lead shield. They were counted under light ether anesthesia according to the method of Wolff (6). A rate meter, recording impulses from the same detector, was used to center the tumors. Correction for body background, because of changes in equipment, was different than that previously employed. It was found empirically that subtraction of twice the body background (measured over the lower chest) from the combined counts in the right and left tumors gave results within 10% of those obtained by counting the tumor after removal from the animal. For reasons not entirely clear, the best correction after SCN^- treatment was obtained by subtracting only $1\times$ the body background from the pooled right and left tumor counts. Thiocyanate was administered intraperitoneally as shown in Figure 3.

To determine the extent of organification, tumors were removed at various intervals after I^{131} injection and ground with cold 10% trichloracetic acid, 2 ml. per 200 mg., centrifuged, and washed three times with 2.5 ml. of cold 5% trichloracetic acid; or, tumors were homogenized with propylthiouracil solution ($3\cdot10^{-4}M$) and chromatographed in n-butanol:acetic acid:H_2O (75:10:15), n-butanol equilibrated with 2N NH_4OH, or n-butanol:dioxane (80:20) equilibrated with 2N NH_4OH. Salt fractionation and ultracentrifugal analysis of tumor extracts 3 days after I^{131} injection were carried out as described in a subsequent report (7). The very small fraction of radioactivity that became incorporated into protein was hydrolyzed with crude pancreatin in Tris buffer at pH 8.4 for 48 hours at 37° C and chromatographed in the above solvents.

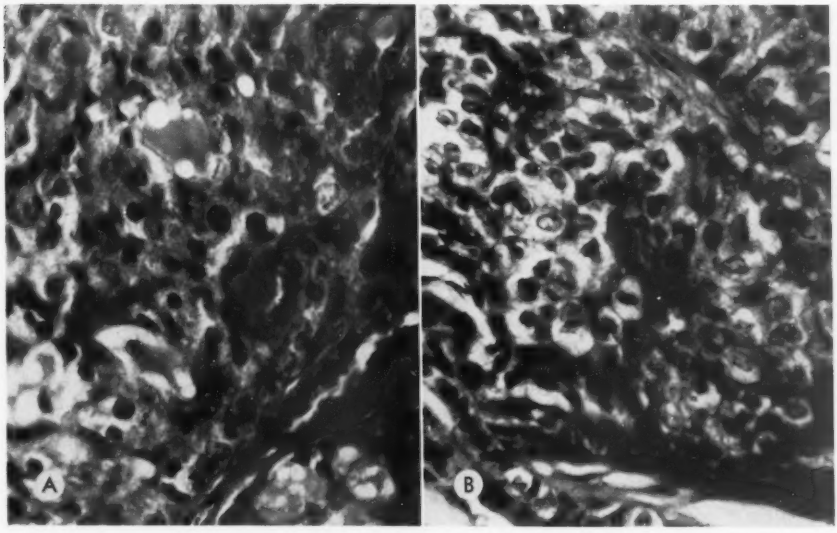
Tumors studied *in vitro* were sliced free hand and incubated in Krebs-Ringer phosphate medium under air or O_2 plus the additions noted. No differences were noted between the use of air or O_2 as the gas phase. The tumor slices were so friable that it was found necessary to centrifuge all media briefly to remove debris in order to calculate the T/M ratio. All values are expressed on the basis of the final weight of the slices. When sheep thyroid slices were used for comparison, they were prepared in a similar manner and labeled *in vitro* in the presence of $1–3\times10^{-3}M$ 1-methyl-2-mercapto-imidazole.

The 2,4-dinitrophenol was twice recrystallized from acetone. Dicumarol was obtained from the Mann Research laboratories, New York, N. Y. and was used without further purification. KSeCN was supplied by the Bios Laboratories, New York, N. Y. The thyrotropin (TSH) was either the Armour product or was kindly supplied by Dr. Peter

Condliffe. Crystalline glutamic dehydrogenase was obtained from Boehringer & Sons, and TPN and TPNH from the Sigma Co.[2]

RESULTS AND DISCUSSION

Tumors harvested between five and ten weeks after transplantation ranged in weight from about 3.5 to 28 gm. for rats weighing in the neighborhood of 150 gm. This histologic appearance varied from a fairly well differentiated type with preservation of the follicular pattern but non-uniform arrangement of cells (Fig. 1a), to a poorly differentiated type without follicles in which the cells were arranged in small, solid nests (Fig. 1b). The nuclei varied considerably in size and location within the cell. The



FIGS. 1a and 1b. Thyroid tumors from rats of Line 1-5A. Hematoxylin and eosin (×485)

histological appearance is consistent with adenocarcinoma of the thyroid. Older tumors tended to have necrotic areas and occasional cysts filled with clear yellow fluid.

In vivo studies

Following the intraperitoneal injection of radioiodide, the maximum I^{131} content of the tumors was found at 0.5 to 2.5 hours and amounted to approximately 40 to 70% of the injected dose (Fig. 3). The I^{131} content was roughly proportional to the size of the tumor. Radioactivity rapidly dis-

[2] Abbreviations used are TPN for triphosphopyridine nucleotide, TPNH for reduced triphosphopyridine nucleotide, TSH for thyrotropin, T_3 for L-3,5-3′-triiodothyronine, DNP for 2,4-dinitrophenol, T/M for tissue:medium concentration ratio of I^{131}.

appeared from the tumors with a half-life of approximately 4–8 hours (Fig. 2).

Early peaking of thyroidal radioactivity followed by a rapid decline suggested blocking of organic binding in the thyroid tissue similar to effects produced by propylthiouracil (8). Analysis of tumor tissue for iodide and

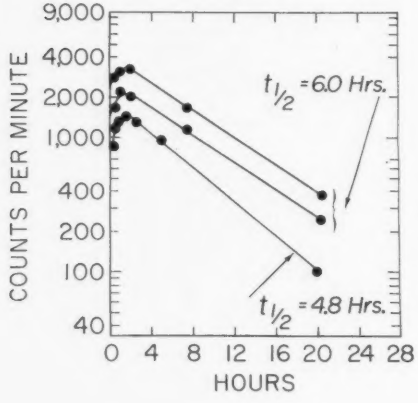


FIG. 2. I^{131} accumulation and release *in vivo* in three thyroid tumors of Line 1–5A. 1.5 μc. of carrier-free I^{131}-iodide were injected intraperitoneally at zero time.

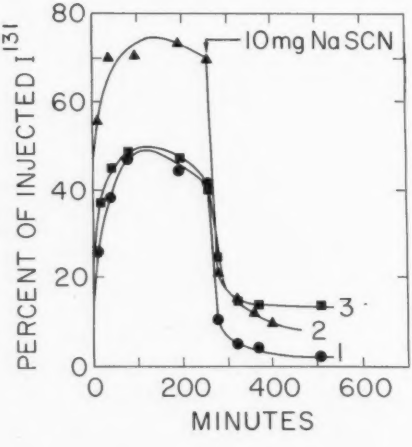


FIG. 3. The Effect of NaSCN on tumor I^{131} *in vivo*. 10 μc. of carrier-free I^{131}-iodide were injected intraperitoneally at zero time. NaSCN was injected intraperitoneally after 260 minutes.

organic iodine revealed that nearly all of the radioactivity present behaved like iodide (Table 1). The non-iodide radioactivity did not behave as thyroglobulin, but was found in the "particulate" fraction of the tumor (7). It yielded, upon pancreatin hydrolysis at pH 8.4 for 48 hours: 36% monoiodotyrosine, 4% diiodotyrosine, 26% iodide, 9% organic material and 25% unidentified radioactivity near the solvent front.

If at least 98–99% of the tumor radioactivity was iodide, then this should be discharged by the administration of SCN^-. As shown in Fig. 3, the major portion of the I^{131} was, in fact, discharged by the intraperitoneal in-

jection of 10 mg. NaSCN. Of the remaining radioactivity 98.6% was trichloracetic acid soluble (Table 1) which compared favorably with the value of 98.2% for the I^{131} of livers of the same animals.

Although Wollman and Reed (5) had shown that this tumor was no longer dependent *for growth* upon antithyroid treatment of the host, it was of some interest to see if there remained a functional dependence upon the level of available thyrotropin (TSH). Accordingly, the "uptake" and release curves of the tumors of six rats were measured during control conditions, and subsequently after treatment with two daily subcutaneous injections of 15 U.S.P. units of TSH for two days. In a third experiment on three of these six rats, 5 μg. of L-triiodothyronine were injected intraperitoneally twice a day for a total of seven injection, following which "uptake"

TABLE 1. THE DISTRIBUTION OF RADIOACTIVITY IN TUMOR TISSUE *in vivo*

Time after I^{131} injection	Per cent of iodide	Method of analysis
1 hour	99.2	TCA soluble fraction
8 hours*	98.6	TCA soluble fraction
21 hours	98	Chromatography† Dialysis‡
3 days§	95–99	Chromatography Homogenization & Extraction

* 3½ hours after most of the I^{131} was discharged by SCN^-—see fig. 3.
† Average values with three solvents: Butanol:acetic acid:H_2O, butanol:ammonia(2N), butanol:dioxane:ammonia(2N).
‡ 1-ml. of homogenized tissue vs. 6L of water.
§ These animals had been thyroidectomized 19 days prior to study in order to obviate inclusion of material of thyroid origin.

and release curves were again performed. The results, depicted in Fig. 4, demonstrate quite clearly that alterations in thyrotropic stimulation by large doses of either TSH or triiodothyronine failed to alter significantly either the maximum amount of radioactivity found in the tumor, or the release rate (biological half-life) of the accumulated I^{131}. That the TSH used was an active preparation is shown by the increased I^{131} content of the thyroids of these rats. The considerable but unchanging I^{131} in the thyroids of the triiodothyronine treated rats can be accounted for by residual radioactivity from the previous experiment.

In vitro studies .

Optimal iodide trapping could be demonstrated without the addition of an antithyroid agent, hence the present system offered an unusually good opportunity to study the trapping mechanism. Except for old tumors, which exhibited considerable necrosis and low iodide concentrating ability, the *in vitro* concentration gradients (T/M ratios) varied from 8 to 44 in slices of the tumor for five generations. Equilibration with the medium

occurred at 30–60 minutes at 37°C and most determinations were carried out at 60 or 90 minutes. The equilibrium value was maintained for several hours and then gradually declined.

Intact cell structure appeared to be required since equilibrium dialysis through "Visking" sausage casing of a 20% homogenate of the tumor (in 0.25 *M* sucrose) for periods up to 48 hours at 4° C yielded a gradient of only 1.01 in favor of the protein containing compartment. Ultrafiltration through the same membrane yielded a mean gradient of 1.10.

A variety of agents has been shown to be able to reduce or abolish iodide concentration in normal thyroid tissue (9, 10). A similar effect was exerted by these agents in slices of this tumor. Uncoupling agents, such as 2,4-dinitrophenol or dicumarol, markedly reduce the T/M ratio (Table 2). It is of some interest that thyroxine and triiodothyronine ($1 \cdot 10^{-4} M$), also

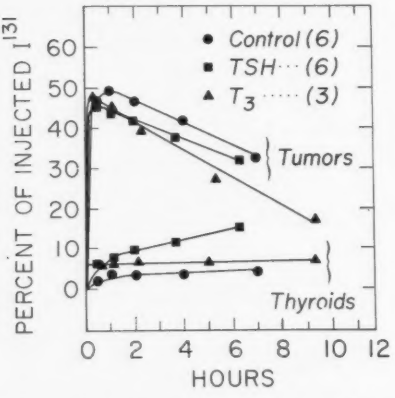


FIG. 4. The Effect of thyrotropin (TSH, 15 U.S.P. units subcutaneously b.i.d.) and L-triiodothyronine (T_3, 5 μg. intraperitoneally b.i.d.) on the accumulation and release of tumor and thyroid I^{131}. Approximately 1.5 μc. of carrier-free I^{131}-iodide were injected intraperitoneally for each period, control, TSH and T_3, at zero time. Numbers in parenthesis indicate number of rats used.

known to uncouple oxidative phosphorylation in mitochondria of liver or kidney at these concentrations (11), failed to lower the T/M ratio in tumor or sheep thyroid slices. Triiodothyroacetic acid ($10^{-4} M$) had a slight depressing effect. The failure to lower the T/M ratio may be due to permeability effects. The possibility that the divalent cations Mg^{++} or Ca^{++} would bind the available thyroxine or triiodothyronine was ruled out by omission of these ions. Under these conditions, control T/M ratios were only slightly lower and they were not altered by the thyroid hormones. The possibility that thyroid tissue is not sensitive to uncoupling by its own products is, however, still being considered.

Thyrotropic hormone at rather high levels did not influence the ability to concentrate iodide in tumor slices and 1-methyl-2-mercaptoimidazole similarly had no augmentative effect (Table 2).

It was shown above that in *in vivo* experiments, thiocyanate ion discharged iodide ion from the tumor. It was thus of some interest to compare the series cyanate (-OCN), thiocyanate (-SCN) and selenocyanate (-SeCN) *in vitro* (Table 2). Telurocyanate was unfortunately not available. The

TABLE 2. IODIDE (I^{131}) CONCENTRATION BY SLICES OF RAT THYROID TUMOR AS INFLUENCED BY VARIOUS AGENTS

Agent	Concentration	Per cent of control T/M ratio*
	M	
2,4-Dinitrophenol	$1 \cdot 10^{-4}$	6
	$1 \cdot 10^{-5}$	48
Dicumarol	$1 \cdot 10^{-4}$	6
	$2.3 \cdot 10^{-5}$	34
	$1 \cdot 10^{-5}$	84
L-Thyroxine	$1 \cdot 10^{-4}$	108
L-Thyroxine ($-Mg^{++}$)	$1 \cdot 10^{-4}$	96
L-Thyroxine ($-Mg^{++}$, $-Ca^{++}$)	$1 \cdot 10^{-4}$	109
L-Triiodothyronine	$1 \cdot 10^{-4}$	105
L-Triiodothyronine ($-Mg^{++}$)	$1 \cdot 10^{-4}$	109
Triiodothyroacetate	$1 \cdot 10^{-4}$	81
Triiodothyroacetate ($-Mg^{++}$)	$1 \cdot 10^{-4}$	74
TSH	1 unit/ml.	86
1-Methyl-2-mercapto-imidazole	$1 \cdot 10^{-3}$	97
KOCN	$3 \cdot 10^{-4}$	100
KSCN	$3 \cdot 10^{-4}$	10
KSeCN	$3 \cdot 10^{-4}$	9
$KClO_4$	$3 \cdot 10^{-4}$	8

* Each value represents the mean of at least three flasks. Since different tumors with different control T/M ratios were used (range: 13 to 26) values are expressed as per cent of the appropriate control.

oxygen analogue failed to discharge significant quantities of iodide from the tumor slices, whereas both the thio- and seleno-cyanates were very active.

The isolation of the trapping mechanism with a nearly complete block in the further metabolism of iodine led us to speculate on the missing step. It was possible that the ability to synthesize the required substrate, thyroglobulin, had been lost. Ultracentrifugal analysis of saline extracts of the tumor (Fig. 5) revealed material with a sedimentation coefficient like that of thyroglobulin ($S_{20} = 19$) (7). Although the amount was much less than that generally found in saline extracts of normal thyroid tissue, it was of the same order as that found in another transplantable rat thyroid tumor (Line 1–8) in which iodination of a protein indistinguishable from normal rat thyroglobulin readily occurred. The fraction obtained by salting out between 1.4 *M* and 1.75 *M* potassium phosphate buffer was enriched in protein with a behavior characteristic of thyroglobulin (Fig. 6). While it is possible that the protein found here was abnormal in a subtle manner not detected by these methods, it appears likely that uniodinated thyroglobu-

FIG. 5. Schlieren pattern of saline extracts of two rat thyroid tumors, run simultaneously in the Spinco Model E ultracentrifuge at 59,800 rpm. The extracts were dialyzed against a large volume of .001 *M* potassium phosphate buffer, pH 6.5, lyophylized, and dissolved in .15 *M* NaCl at a concentration of 10 mg. per ml. The *upper curve* is the material from tumor line 1–8 (cf. ref. 7) in a cell with one prismatic face. The *lower curve* is the material from tumor line 1–5A in a standard cell. The sedimentation coefficients given in Svedbergs were identical for the two extracts. The apparent difference in displacement of the peaks is due to the difference in position of the menisci. The area under the 19S peak is 4% of the total in the upper curve, 6% in the lower.

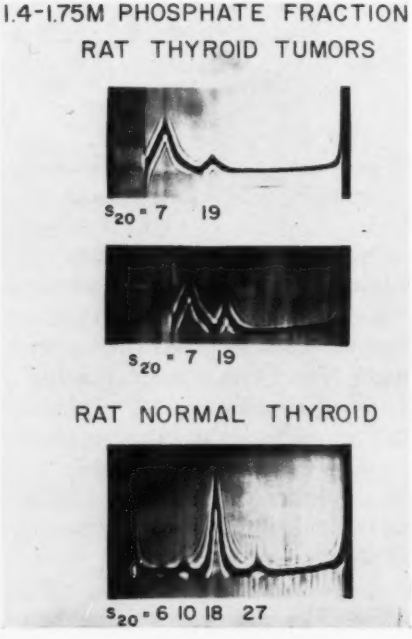


FIG. 6. Schlieren pattern of fractions obtained from saline extracts of two rat thyroid tumors and one normal rat thyroid run in the Spinco Model E ultracentrifuge at 59,800 rpm. Each fraction is that portion of the extract precipitating between the limits of 1.4 *M* and 1.75 *M* potassium phosphate buffer, pH 6.5 (cf. ref. 7). Each fraction was dialyzed against a large volume of .001 *M* potassium phosphate buffer, lyophylized, and dissolved in 0.15 *M* NaCl at a concentration of 4 mg./ml. The *upper curve* is from rat tumor line 1–5A. The *middle curve* is from rat tumor line 1–8. The sedimentation coefficients are given in Svedbergs. The area under the 18–20S peaks are 18% of the total in the upper curve, 27% in the middle curve, and 81% in the lower curve.

lin *was* present and that the defect was in the iodination system and not in its substrate, thyroglobulin.

Another system of importance to the iodine economy of the normal gland is the deiodinating system for free iodotyrosines (12) recently described in detail (13). While no apparent "need" would exist for such an enzyme in a tumor that has lost practically all of its ability to synthesize organic iodine compounds, the possibility nevertheless existed that this function had not been lost. As can be seen from Table 3 there was essentially no L-diiodotyrosine deiodinating activity in this tumor. Even though we were unable to prepare normal homogenates quite as active as those of

TABLE 3. DEIODINATION OF L-DIIODOTYROSINE BY THYROID TUMOR TISSUE

Tissue	mμmoles of iodide liberated from diiodotyrosine
Tumor slices	<0.1
Tumor homogenate	0.4
Tumor homogenate (boiled)	0.2
Tumor homogenate + TPNH*	0.1
Sheep thyroid homogenate	3.6

All flasks contained 13.8 mμmoles of L-diiodotyrosine labeled with I^{131}. 165 mg. of slices were incubated in 3 ml. of Krebs-Ringer phosphate solution. Homogenates were prepared in 0.38M sucrose and an amount equivalent to 250 mg. of fresh tissue was added to Krebs-Ringer phosphate (pH 7.4) to a final volume of 3.0 ml.

* Generating system—250 μg. crystalline glutamic dehydrogenase, 0.24 μmoles TPN, 2 μmoles nicotinamide, 2 μmoles glutamate; or 0.54 μmoles TPNH and 2 μmoles nicotinamide.

Stanbury (13) it seems safe to conclude that nearly all of this activity had disappeared from the tumors. Furthermore, a loss of TPNH-generating ability, which may have occurred in the tumors, could not explain the absence of deiodinating activity, since addition of TPNH, or a very active preparation of glutamic dehydrogenase with TPN and glutamate, failed to restore activity (Table 3).

COMMENTS

It appears safe to conclude that the transplantable thyroid tumor described above represents an iodide concentrating mechanism that is isolated from the remaining pathways of iodine metabolism by a defect in the ability to form organic iodine. The question now arises as to the normalcy of the "trap" in this tumor. The normal range of the T/M ratio, the rapid discharge of I^{131} from the tumor *in vivo*, the chemical behavior of the I^{131} as iodide, and the response to various inhibitors all are properties indistinguishable from the "trap" of normal thyroid tissue. It is tempting to postulate therefore, that the lack of response *in vivo* to alterations of the TSH level described above must mean that the trophic hormone does not influence the iodide concentrating mechanism directly, but does so *via* other factors. VanderLaan and Caplan (14) and Halmi and Spirtos (15) have suggested that reduction in organic thyroid iodine enhances the thy-

roid iodide "trap" independently of TSH. As the present tumor has very little, if any, hormone iodine in it to begin with, such a mechanism could not be operative. TSH therefore, (if depression of thyroid organic iodine were the sole mechanism by which it influences iodide concentration) would be ineffective in this tissue. There are, however, data to suggest that even in a situation in which TSH is unable to change the organic thyroid iodine content, it may still enhance iodide trapping. VanderLaan and Greer (16) and Taurog *et al.* (17) have shown that in propylthiouracil treated rats whose thyroids were depleted of iodine, hypophysectomy reduces the T/S ratio, and the latter workers further showed that subsequent TSH elevated thyroid iodide concentration.

All the properties of the "trap" in the tumor, however, may not be identical with those of normal thyroid tissue. One difference has been detected between the iodide trap in the tumor and in normal thyroid tissue. It has

TABLE 4. INHIBITION OF IODIDE TRANSPORT BY CARDIAC GLYCOSIDES

	Concentration for 50% inhibition of the T/M ratio*	
	Sheep thyroid slices	Rat tumor slices
	M	M
Digitoxin	2×10^{-7}	$\sim1.5\times10^{-4}$
Strophanthidin	5×10^{-7}	$\sim 9\times10^{-4}$

* Incubated at 37° C under air in Krebs-Ringer phosphate buffer pH 7.4 for 70–90 minutes.

recently been shown (18) that a variety of cardiac glycosides or their aglycones, such as strophanthidin, which are known to interfere with cation and anion transport, (19, 20, 21) can block iodide accumulation by normal thyroid tissue. As can be seen in Table 4, the iodide "trap" in slices of this tumor was approximately 1000 times less sensitive to the cardiac glycosides than that of normal sheep thyroid slices. Since the iodide "trap" in this tumor differs in at least one respect from that in normal sheep thyroid tissue, it may not be justifiable to draw conclusions about the normal "trap" from the present experiments.

Acknowledgment

We should like to express thanks to Dr. S. H. Wollman for supplying the original tumor, to Dr. Leon Sokoloff for preparing the histological material, to Mr. J. R. Maurey for technical assistance, and to Kilsheimer Bros., Inc. for making sheep thyroid glands available.

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IODOPROTEINS IN THYROID TISSUE AND BLOOD OF RATS WITH A TRANSPLANTABLE THYROID TUMOR[1]

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ABSTRACT

Iodoproteins other than thyroglobulin have been detected in a transplantable rat thyroid tumor (Wollman) in which iodine compounds were labeled with I^{131} either *in vivo* or *in vitro*.

Two types of iodoprotein were found. One was not extractable from glass homogenized tissue by 0.15 *M* NaCl, and is termed "particulate iodoprotein," or "P-1." In *in vivo* experiments, it increased with time after I^{131} administration from about 15% to about 70% of the total organic iodine in the tumor. Cell particle fractionation identified this material with the "nuclear" fraction.

The second type of iodoprotein was readily extracted from previously frozen tissue slices. Unlike thyroglobulin, it was soluble in 1.89*M* $KH_2PO_4-K_2HPO_4$, sedimented slowly in the ultracentrifuge, and appeared multidisperse on zone electrophoresis. This iodoprotein fraction comprised 3 to 9% of the soluble iodoproteins, the remainder being thyroglobulin, and its amount relative to thyroglobulin did not change with time after I^{131} administration. It has been termed "thyroid S-1 iodoprotein."

Rats bearing the thyroid tumor were also found to have iodoprotein circulating in the blood. Characterization of this material revealed it to be similar to thyroid S-1 iodoprotein, but perhaps not identical. It has been termed "serum S-1 iodoprotein."

All of these iodoproteins could be hydrolyzed by a crude pancreatic enzyme mixture, with the liberation of iodinated amino acids of the type found in thyroglobulin. When compared to thyroglobulin in the same tumor, however, they had lower iodinated thyronine:tyrosine ratios, and the thyroid and serum S-1 iodoproteins had lower diiodotyrosine:monoiodotyrosine ratios as well. The particulate iodoprotein also had a higher triiodothyronine:thyroxine ratio compared to thyroglobulin.

Time studies, while inconclusive, did not support the possibility that P-1 iodoprotein might be a precursor of thyroglobulin. It appeared likely, however, that thyroid S-1 iodoprotein was the precursor of serum S-1 iodoprotein.

COMPARATIVELY little attention has been given to naturally occurring vertebrate iodoproteins. The earlier work on this subject, and more recent experiments by Derrien and co-workers, have been reviewed by Roche and Michel (1). They concluded that "the normal gland synthesizes only a single substance for iodination. Different thyroglobulins exist only among different animal species and in abnormal organs but each of these thyroglobulins may be more or less iodinated." Since that time, only

[1] Presented in part at the 1958 Meeting of The American Goiter Association.

a few reports have appeared on this subject. Shulman, Rose and Witebsky have compared the proteins (but not necessarily iodoproteins) in extracts of normal and abnormal thyroid tissue by ultracentrifugal and electrophoretic analysis (2, 3), and have isolated a protein fraction which is more soluble in ammonium sulfate than thyroglobulin, and which they have designated "thyralbumin" (4). Recently the fraction containing "thyralbumin" was shown to also contain iodine (5). Stanley and co-workers described differences in the salting out of iodine in normal and abnormal thyroid tissues (6), and found an apparent difference in electrophoretic mobility of thyroglobulin isolated from normal and abnormal tissue in one human subject (7). Immunological differences have been noted in thyroid proteins derived from different species (8, 9) and from normal and abnormal thyroid tissue in the same subject (3). Roche and co-workers (10) found that the salting out curves of thyroid iodoproteins were identical in a normal subject and in patients with Graves' disease.

Iodoprotein has not been found in the blood of normal individuals, although the circulating iodine, largely in the form of thyroxine, is reversibly bound to certain serum proteins (11). An iodoprotein with the immunochemical and/or physical characteristics of thyroglobulin has, however, been detected in blood after surgical manipulation (12) or radiation therapy (13) of thyroid tissue. Iodoproteins have also been found in the blood of human subjects with various thyroid disorders such as thyroid carcinoma (14, 15), thyroiditis (16, 17, 18), congenital goiters (19, 20), and hypothyroidism (21, 22). In a few reports (14, 15, 20) this material has been characterized sufficiently to differentiate it from thyroglobulin and from iodinated serum protein. In animals, iodoprotein has been demonstrated in thyroid vein blood in the calf (23) and in thyroid lymph in the dog (24), but its identity was not established.

This report presents the findings in a study of iodoproteins in thyroid tissue and blood in rats bearing a transplantable thyroid tumor which has the ability to form organic iodine. The accompanying paper (25) presents similar studies on normal and abnormal thyroid tissue in human subjects and in normal sheep thyroid. In all instances, iodoproteins other than thyroglobulin have been observed.

MATERIALS AND METHODS

The thyroid tumor employed throughout these studies was one of a series of thyroid tumors developed in Fischer rats by Wollman and Reed (26). Initially resulting from the administration of thiouracil to normal animals, this tumor had, in the process of serial subcutaneous transplantation, become autonomous. The tumor selected for this study (designated Line 1–8 in Wollman's classification) retained a high degree of function both in accumulation and organification of iodine. Indeed, this tumor secreted sufficient hormonal iodine to produce suppression of iodine accumulation by the host's normal thyroid gland, presumably as a result of a suppression of pituitary thyrotropic secretion. The protein-bound iodine (PBI) measured in serum or plasma of five of the animals

under study ranged from 5.6 to 17 μg. per 100 ml.[2] (Normal plasma PBI in Fischer rats was found to be 1.6 to 2.4 μg. per 100 ml.). The butanol-extractable iodine (BEI) in three tumor-bearing animals, however, was 2.1 to 2.9 μg. per 100 ml. (cf. below).

All of the tumors used were derived from a single tumor (Wollman's line 1–8, generation 12) which was carried by subcutaneous implantation through three generations of female rats during the course of this study. The tumor did not appear to undergo significant alteration in function during this time. The tumors were harvested approximately 3 to 4 months after implantation, when they had attained an average size of about 7 gm. In most instances the normal thyroid gland of the host was excised at least 3 days prior to study of the tumor or blood. The animals were maintained on a diet of Purina laboratory chow, *ad lib.*, with added greens once each week.

The iodine in tumor and blood was labeled with I^{131} and all studies were carried out on the radioactive material. In most instances, labeling was by *in vivo* incorporation of radioiodide administered intraperitoneally. In a few experiments, labeling was done *in vitro* by incubation of tissue slices, approximately 0.2 to 0.5 mm. thick, with radio-

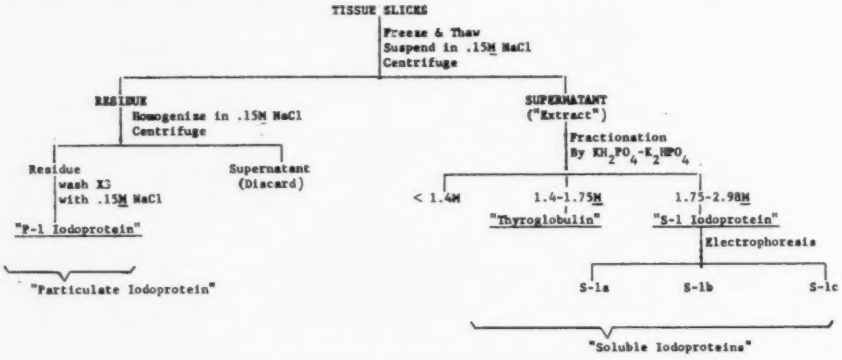

FIG. 1. Scheme for tissue fractionation.

iodide in an oxygenated Krebs-Ringer phosphate medium pH 7.4, at 37° C in an oscillating water bath.

In the *in vivo* experiments, the animals were anesthetized with ether and killed by exsanguination. Tumor tissue was frozen and sliced with a microtome at a thickness of approximately 0.2 to 0.5 mm. The slices were then suspended in 0.15 *M* NaCl (usually in a ratio of 1 gm. of slice to 3 ml. of fluid) at 4° C for 16 to 24 hours. Slices after *in vitro* iodination were similarly suspended after freezing. The supernatant extract after low speed centrifugation was used for the study of the soluble iodoproteins. The residue was then resuspended in 0.15 *M* NaCl and homogenized in a glass homogenizer of the Potter-Elvehjem type. The supernatant after centrifugation in an angle centrifuge at 12,000 to 20,000 rpm for 30–45 minutes was usually discarded. In several experiments, however, the radioiodine in this fraction was examined and found to resemble that in the initial saline extract except for a lower iodide content. The residue was then resuspended, washed 3 times with 0.15 *M* NaCl, and used for the study of insoluble, or particulate, iodoproteins. In some experiments, the saline extraction step was omitted, the fresh tissue was immediately homogenized, and the first supernatant was used to study the soluble iodoproteins. The fractionation scheme is outlined in Figure 1.

[2] PBI was determined by the method of Zak, *et al.* (27) by the Boston Medical Laboratory, Boston, Mass. Two of the PBI measurements were done on plasma from thyroidectomized rats. All other I^{127} measurements were done on intact animals.

In those experiments in which the particulate fraction was subfractionated, a separate portion of tissue was homogenized without freezing in .88 *M* sucrose in a ratio of 1 gm. to 10 ml. Fractionation by differential centrifugation into "nuclear," "mitochondrial," and "microsomal" fractions was carried out according to the procedure of Hogeboom and Schneider (28). After initial analyses with this technique, the method of Hogeboom, Schneider and Striebech (29) was used for the preparation of the "nuclear" fraction. In this method, the homogenate prepared in 0.25 *M* sucrose containing 1.8×10^{-3} *M* $CaCl_2$, was layered over 0.34 *M* sucrose containing 1.8×10^{-4} *M* $CaCl_2$ and subjected to centrifugation at $600\times G$ for 10 minutes. The precipitate at the bottom of the denser layer was rehomogenized in sucrose and the above procedure repeated three times.

ANALYTICAL METHODS

Salting out curves of soluble iodoproteins in tissue extracts and in serum were performed with equimolar KH_2PO_4-K_2HPO_4 at room temperature by the method described earlier (13). An amount of extract or serum sufficient to permit radioactivity measurements was added to diluted normal human serum, the latter providing carrier protein for removal of precipitates. The final serum dilution was 1:30; the amount of tissue protein was variable, but very much lower. In two instances batch fractionation with phosphate buffer[3] was also carried out between the limits of salt concentration indicated under Results. Carrier serum proteins were not added in these experiments, and ether, which was used in the salting out curves to facilitate separation of the precipitate, was omitted. Precipitates were removed by centrifugation and redissolved. The fractions were dialyzed at 4° C through Visking tubing against large volumes of approximately 10^{-3} *M* phosphate buffer, and lyophylized.

Ultracentrifugal analyses in which the I^{131} labeled substances were measured were done with the Spinco Model L instrument at 39,000 RPM (Rotor No. 40) for 2 or 3 hours. The liquid in the tube was sampled stepwise from the top downward by means of a syringe and needle mounted on a rack and pinion. Optical ultracentrifugal analyses were done with the Spinco Model E instrument by the usual methods. In most runs, a cell with one prismatic face was used in place of the counterbalance, so that 2 solutions could be examined simultaneously.

Zone electrophoresis in filter paper was performed in the conventional manner, and with the reverse flow technique, as described earlier (30). Chromatography on ascending paper strips (Whatman 3 MM) was done in the following solvent systems: n-butanol-glacial acetic acid-water (75-10-15), n-butanol equilibrated with 2N NH_4OH (equal parts), and n-butanol-3,5-dioxane (80-20) equilibrated with 2N NH_4OH (100). Each sample analyzed by chromatography was run in at least two, and usually in all three solvent systems. The values obtained in the various solvents agreed well with each other, in most instances, and the averages are given under Results.

Radioactivity on the paper strips was measured with a mica-window Geiger-Mueller tube and a continuously recording counting-rate meter. The zones of radioiodine were quantitated by planimetry of the areas under the curve. Carrier thyroxine, 3′,3,5-triiodothyronine, 3-monoiodotyrosine and 3,5-diiodotyrosine were placed on each strip together with the sample for localization of the radioactive zones. The carriers were located after scanning-by spraying the strips with a freshly prepared mixture of 4.5% $NaNO_2$, .05*M* sulfanilic acid in 9% HCl, and 2.5% Na_2CO_3 (1:1:8). The first two components of the spray were mixed several minutes prior to addition of the Na_2CO_3.

Localization of the radioactive zones was also aided by preparing autoradiograph of the scanned but unsprayed strips, using no-screen x-ray film. In many instances, further

[3] The term "phosphate buffer," as used in this paper, refers to equimolar KH_2PO_4-K_2HPO_4,.

identification of radioactive components was provided by autoradiography of two-dimensional chromatograms. The first two solvents listed above were employed, with the alkaline system as the first dimension.

Radioactivity assays other than those of paper strips were done with a well-type scintillation counter, employing a NaI crystal.

Hydrolysis was performed with crude pancreatin, U.S.P., in Tris (hydroxymethyl) aminomethane buffer, pH 8.5, at 37° C for approximately 48 hours.

Other techniques employed are described under Results.

RESULTS AND DISCUSSION

The histological appearance of the tumor, and the localization of radioiodine by autoradiography, are illustrated in Figure 2. Radioiodine was associated only with areas of the tumor which showed follicle formation, and the pattern of localization suggested association with the follicles. The methods could not distinguish, however, between radioiodine located in cells or in colloid.

Details of the *in vivo* experiments with regard to the amount of radioiodine, the time of study, and the tumor size and iodine accumulation are listed in Table 1. "Rat No." identifies the individual animals in this and subsequent tables. The data do not permit accurate estimation of the radiation delivered to the tumors. It may be calculated, however, that an average-sized tumor weighing 7 grams and accumulating 50% of a 400 μc. dose, with a biological half-time of 2 days for tumor iodine, would receive an integral dose of approximately 800 rads. The smaller tumors (4 gm.) would receive 1400 rads under similar circumstances. This dose is consid-

TABLE 1. RADIOIODINE IN THYROID TUMORS LABELED *in vivo*

Rat No.	Time after dose	Dose of I^{131}	Tumor weight	Total I^{131}	Organic I^{131}	Particulate iodoprotein	"Nuclear" Fraction*
		(μc.)	(grams)	(% of dose)		(% of organic I^{131})	
1	7+ min.†	320	5.5	8.1	3.3	17	12
2	30	250	4.5	25	21	14	14
3	3 hr.	250	13.5	49	47	16	18
4	7	440	8.6	47	46	(28)‡	
5	1 day	440	7.1	27	27	(32)	
6§	1	230	22	38	30		
7	2	240	5.1	32	32	20	
8	2	440	4.2	31	31	29	
9	2	440	5.0	35	35	39	
10	4	6	8.6	28	‖	(38)	
11	7	440	6.7	5.8	5.8	(62)	
12	7	500	4.5	17	17	44	35
13	12	500	4.0	5.8	5.8	62	58
14	14	46	4.9	6.0	‖	56	31
15	19	500	0.6	0.9	0.9	72	77

* Two-phase sucrose procedure.

† Anesthetized at 5 min., exsanguinated at 7½ min., tumor fractionation begun at 25 min.

‡ Values in parentheses refer to radioiodine in slices which had been extracted with .15 *M* NaCl and then washed 3 times, but not homogenized. It was found that such values are 6 to 36% higher than the particulate iodoprotein values obtained by homogenization.

§ This was the only animal not thyroidectomized prior to study. The thyroid gland weighed 9 mg. and contained 0.7% of the I^{131} dose.

‖ Chromatography omitted.

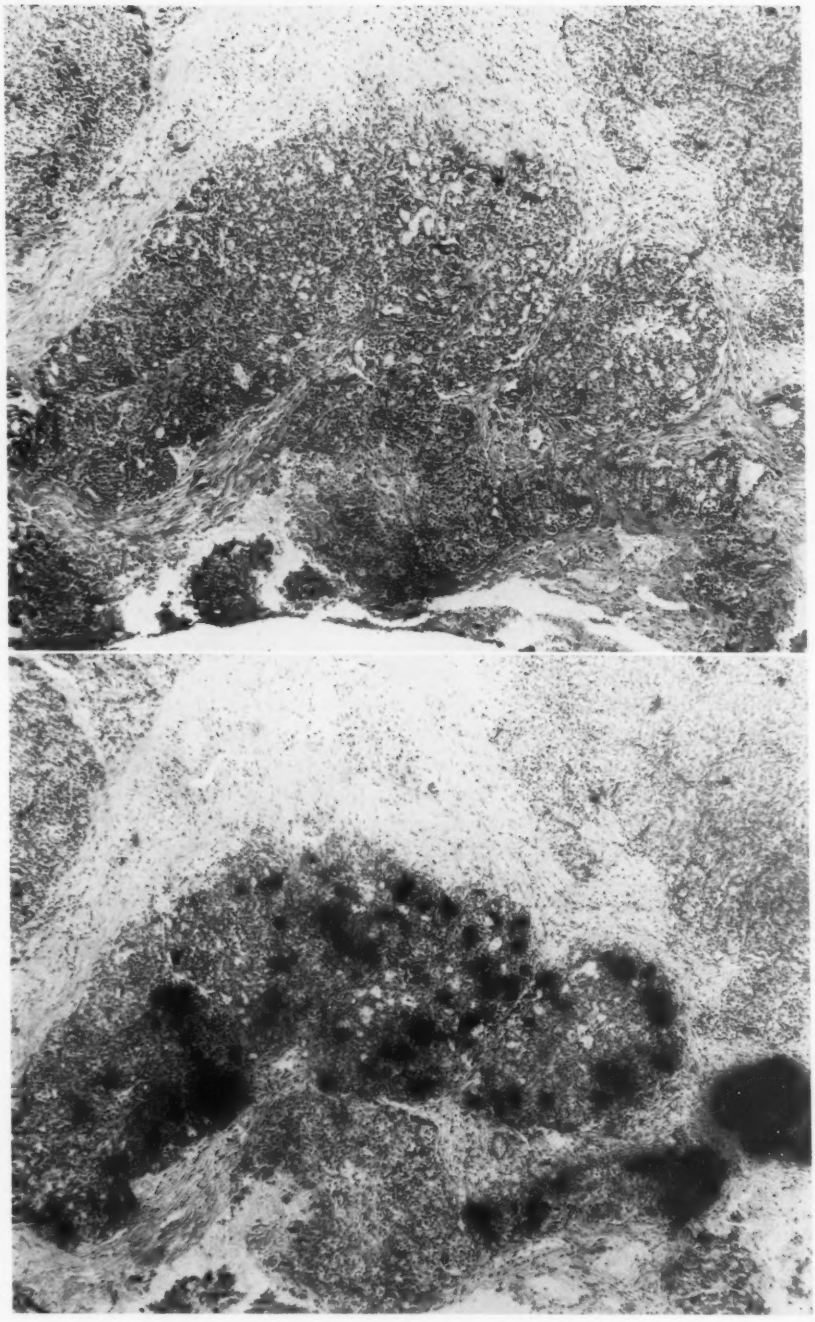

FIG. 2. Histological section and autoradiograph of a thyroid tumor, Line 1-8 (rat no. 10). Hematoxylin and eosin, 70×.

erably sma ler than that which will cause thyroid tumors to discharge thyroglobulin into the blood in man (31). In rat no. 14, in which the dose was 46 μc. and the tumor weighed 4.9 gm., the tumor dose would be only 14 rads. In only one animal (rat no. 15) was the tumor seen to decrease in size following radioiodine administration.

PARTICULATE IODOPROTEIN

The quantity of tumor radioiodine in organic form and the proportion in the non-extractable, or particulate, fraction are given in Table 1 for the *in vivo* experiments. Iodide was determined by chromatography of the tumor extracts. The unhydrolyzed particulate fractions contained no iodide. Iodoproteins in the extracts and in the particulate fractions remained at the origin of the chromatograms, and accounted for almost all of the non-iodide radioactivity. Other radioactive components were sometimes detected. In the unhydrolyzed tumor extracts, zones of radioiodine coinciding with monoiodotyrosine or diiodotyrosine comprised less than 1% of the radioactivity. Trace amounts of radioiodine were sometimes present at the solvent front. In the particulate fractions, the only radioiodine component other than origin material was found at the solvent front. This was present in trace amounts, if at all, except in rat no. 1 in which it comprised 8% of the particulate radioiodine. In the *in vitro* experiments, however, the front-running component was present consistently and in larger amounts (cf. below).

The proportion of tumor radioiodine in the particulate fraction increased with time after the dose from about 15% to more than 70% of the accumulated organic iodine. Normal rat thyroid, by comparison, contained 5% of its radioiodine in this fraction 2 days after the dose. (In this experiment, 8 normal Fischer rats were each given 10 μc. of I^{131}, and the thyroid glands were pooled.)

Whereas radioiodine (as percent of dose) in the soluble fraction of the tumors reached a peak at 3 hours after the dose, the particulate radioiodine (as percent of dose) increased more slowly, reaching a peak at 2 days. The particulate fraction also fell off more slowly so that curves depicting radioiodine in the two fractions crossed at a point between 7 and 12 days after the dose.

Identification of the subcellular particles with which the particulate iodoprotein was associated was attempted by the methods of Hogeboom and co-workers (cf. Materials and Methods). The results of the method employing a single phase of 0.88 *M* sucrose are presented in Table 2. Soluble radioiodine decreased with time after radioiodine administration. Of the remaining I^{131}, the greatest proportion was in the "nuclear" fraction. The results, however, were somewhat variable, and it is uncertain whether the smaller amounts of radioiodine in the other particulate fractions were the result of contamination with nuclear fragments.

In view of these findings, the two phase sucrose method was employed as a more reliable means of isolating cell nuclei. The results of this procedure, listed in Table 1, demonstrate a fairly good agreement between radioiodine in the "nuclear" fraction and that remaining after saline extraction. In the pooled thyroid glands from normal Fischer rats, 4% of the radioiodine was in the "nuclear" fraction. Examination of these "nuclear" preparations by light microscopy revealed apparently well preserved nuclei which retained the Feulgen staining reaction. A moderate amount of amorphous material which stained pink with eosin was also present, but no other particles were detected in significant amounts.

The protein nature of the particulate radioiodine is indicated by the results given in Table 3. After hydrolysis with pancreatic enzymes, approximately 75% of the particulate radioiodine was identified as iodinated ami-

TABLE 2. SEPARATION OF TUMOR RADIOIODINE INTO SUBCELLULAR FRACTIONS BY THE SINGLE PHASE, .88*M* SUCROSE PROCEDURE

Rat No.	"Nuclei"	"Mitochondria"	"Microsomes"	Combined Supernatants
2	20	16	5	59
3	32	9	3	56
A	47	4	2	47
B	41	6	4	49
12	30	18	8	44
13	60	11	2	27

Rat A and B were each injected with 100 μc. of radioiodide 24 hours prior to removal of the tumor.
All values are per cent of organic I^{131}.

no acids, most of which were iodinated tyrosines. These results will be considered further in a later section.

Solubilization and characterization of the particulate iodoprotein are currently under study and will be the subject of a future report.

SOLUBLE IODOPROTEINS

The saline extract of each of the tumors was studied by the salting-out procedure in order to examine the soluble iodoprotein. A typical curve is presented in Figure 3. Most of the radioiodine salted out sharply at a low buffer concentration in a manner characteristic for thyroglobulin. The solubility of this thyroglobulin was the same as that found in normal rat thyroid, as indicated by the salt concentration at which the salting-out curve had its steepest slope (Table 4). Rat thyroglobulin, however, was slightly less soluble than human thyroglobulin (25). The mean value for the point of steepest slope for thyroglobulin in normal and abnormal rat thyroid tissue ($1.53 \pm .02$ *M*)[4] was significantly different from that in normal and abnormal human thyroids ($1.58 \pm .05$ *M*, $p < .001$).

[4] Mean $\pm$ standard deviation.

TABLE 3. HYDROLYSIS OF IODOPROTEINS IN THYROID TUMORS AND SERUM

Rat No.	O	I	M	D	T_4	T_3	U	O	I	M	D	T_4	T_3	U
	A. Soluble in .15 M NaCl							*B. Particulate*						
1*	4	0	54	43	<1			19	8	52	38			
2*	7	4	61	32	2	<1		17	6	60	34			
3*	6	5	38	44	10	3		17	5	46	41	5	1	
4	4	4	35	45	16	2		19	5	7	41	9	4	34
5	5	5	1	38	22	4	31	16	5	1	46	9	8	33
7	5	4	33	38	24	2		14	5	32	48	7	5	
8	4	8	28	29	28	4		14	5	37	42	7	7	
9	6	3	15	40	22	2	19	14	6	2	48	6	5	31
11	10	8	30	39	22	7		19	7	0	51	5	3	27
12	8	5	32	43	17	2		17	6	34	56	4		
13	12	11	12	42	21			22	6	4	60	8		24
15	22	0	28	27	23			18	7	27	71	3		
	C. Insoluble in 1.92 M Phosphate							*D. Soluble in 1.92 M Phosphate*						
1	8	15	38	54				24	8	81	15			
2	5	2	68	32				15	8	21	17			57
3	8	6	16	53	10		19	30	7	71	29			
4	4	4	30	40	24	4		30	6	66	31	3	<1	
5	2	2	33	29	28	4	5	14	6	5	31	12	6	44
12	8	3	31	40	24			32	3	54	32			
13	18	11	21	56	20			35	11	54	39			
	E. Serum—Immobile in Chromatogram													
4	4	21†	72	15										
5	5	10	76	14	5									
9	4	7	74	16										
11	9	5	69	7										

Definition of symbols: O = I^{131} at chromatogram origin, I = iodide, M = monoiodotyrosine, D = diiodotyrosine, T_4 = thyroxine, T_3 = triiodothyronine, U = unknown (cf. text). Values for O and I are in per cent of total iodoprotein. Values for M, D, T_4, T_3 and U are in terms of per cent of hydrolytic products other than iodide and immobile material. Deviation of the sum of these values from 100 per cent is due to other unknowns.

Part A. = whole thyroid extract. Parts C. and D. = fractions obtained from this extract.

* Tumor extract dialyzed before hydrolysis.

† Serum iodoprotein used for hydrolysis was not completely separated from iodide.

The salting-out curve for the tumor extracts (Fig. 3), however, revealed the presence of another radioiodine component more soluble than thyroglobulin. This component was quantitated by subtracting the proportion of extract radioiodine soluble at 2.98 *M* phosphate buffer from that soluble at 1.89 *M*. The results are given in Table 4 where data from each of the salting-out curves is listed. From 2.7 to 9.4% of the soluble iodoprotein was in this fraction in the rat tumors, whereas none was detected in the normal rat thyroid. In normal human and sheep thyroids (25), however, this component comprised as much as 4% of the soluble iodoprotein. This iodoprotein which is more soluble than thyroglobulin will be referred to as "thyroid S-1" to distinguish it from the soluble iodoprotein which can be characterized as thyroglobulin, and from the iodoproteins present in blood (cf. below and Fig. 1).

In order to characterize the thyroid S-1 fraction further, portions of the saline extracts of two thyroid tumors (rats no. 4 and 5) were fractionated with phosphate buffer, as indicated in Table 5 and Figure 1. The fractions

TABLE 4. SALTING-OUT OF SOLUBLE IODOPROTEINS IN THYROID TUMORS AND THYROID GLANDS

Rat No.	1.19M	1.4M	1.89M	2.98M	S-1	Slope maximum
A. Tumor—In Vivo Label						
1*	100	81.8	8.2	3.7	4.5	1.54
2*	93.7	79.0	11.4	2.0	9.4	1.54
3*	94.8	78.9	6.5	1.6	4.9	1.58
4	93.5	91.2	8.7	4.1	4.6	1.54
5	96.4	90.1	7.9	3.3	4.6	1.54
6	96.8	90.6	27.4	18.3	9.1	1.54
7	94.2	86.1	6.7	2.8	3.9	1.54
8	94.9	75.0	6.1	3.4	2.7	1.54
9	94.6	84.6	8.9	4.1	4.8	1.54
11	90.2	76.8	14.9	7.8	7.1	1.54
12	91.0	83.1	9.4	5.3	4.1	1.54
13	90.1	73.8	13.7	10.6	3.1	1.54
B. Tumor—In Vitro Label						
Exp. 1, 3 Hr*	94.0	65.9	8.8	4.2	4.6	1.47
6 Hr*	91.5	69.9	7.9	1.8	6.1	1.50
C. Rat Thyroid Gland						
—	98.5	86.7	4.0	4.2	−0.2	1.54
6	97.0	71.2	4.8	6.0	−1.2	1.50
—	105	94.5	2.7	2.1	0.6	1.54
—	95.6	82.6	3.7	2.4	1.3	1.50

Columns 2 through 5 give the per cent solubility of the radioiodine in the tissue extract at the indicated concentrations of KH_2PO_4-K_2HPO_4.

Column 6 is the difference between columns 4 and 5.

Column 7 gives the molar concentration of KH_2PO_4-K_2HPO_4 at which the salting out curve of thyroglobulin had its maximum slope.

* Tissue extract dialyzed before salting-out.

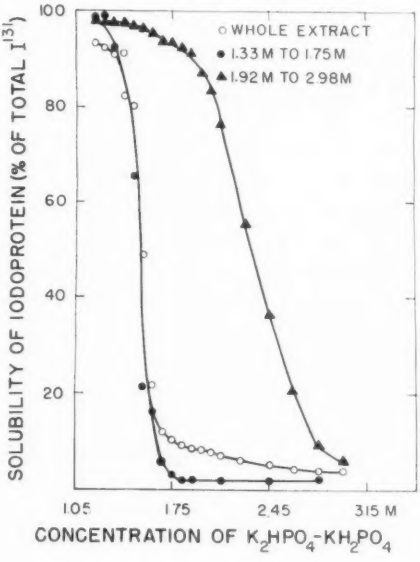

FIG. 3. Salting out of soluble iodoproteins from a thyroid tumor. The solid symbols represent iodoprotein fractions isolated by precipitation between the indicated limits of KH_2PO_4-K_2HPO_4.

TABLE 5. FRACTIONATION OF SOLUBLE IODOPROTEINS IN THYROID TUMORS

Rat No.	Phosphate fraction	% of total tissue weight	% of total extract I^{131}	Thyroglobulin* (% of I^{131} in fraction)
4	<1.33 M	2.1	31	
	1.33–1.75 M	0.3	31	
	1.92–2.98 M	1.7	1.7	4
5	<1.4 M	2.2	32	
	1.4 –1.75 M	0.2	37	
	1.75–2.98 M	1.5	3.2	30

* Determined from salting out curve.

were prepared by single or, at most, two cuts at the concentrations listed. No attempt was made to improve the purity of the fractions by multiple reprecipitation since the quantity of material available was small. Contamination of S-1 with thyroglobulin was, therefore, not unexpected. Furthermore, it is possible that the salting-out characteristics of the tissue proteins in the batch procedure were different from those in the salting-out curves, where carrier serum protein was employed. The recoveries listed in Table 5 must be interpreted in the light of losses incurred in the process of fractionation, dialysis and lyophylization. When whole extract was carried through the last two steps in one rat tumor and in two human thyroids (25) recovery of total soluble iodoprotein was 70 to 75%.

It is of interest that the thyroglobulin fraction in the rat tumors (1.4–1.75 M) contained the smallest amount of material by weight, in contrast to the findings in normal thyroids from sheep and man (25). It seems likely that this difference is correlated with the scarcity of colloid noted histologically in the tumors since total solids in the rat tumor extracts and in the sheep and human thyroid extracts did not appear to be greatly different. It is not known whether there were also differences in colloid composition. Variation in I^{131}-weight ratio among the tumor fractions were most likely due to inclusion of different quantities of non-radioactive material. It is unknown, however, whether the specific activities of the iodoproteins themselves differed.

The significance of the large radioiodine recovery in the fraction less soluble than thyroglobulin is uncertain. This may, in part, be due to denaturation of thyroglobulin during the fractionation, since this fraction contained material which could not be redissolved in 0.15 *M* NaCl. The soluble portion of this fraction resembled thyroglobulin by all of the criteria described below, and the hydrolytic products of the entire fraction resembled those of thyroglobulin. The larger proportion of radioiodine in this fraction in the rat tumors compared to human thyroid (25) is presumably related, in part at least, to the lower solubility of rat thyroglobulin.

Chromatographic analysis revealed that all of the radioiodine after fractionation was still immobile. Comparison of thyroglobulin and the fraction

more soluble than thyroglobulin by salting-out, ultracentrifugation and electrophoresis are illustrated in Figures 3, 4 and 5. The results for one of the tumors (rat. no. 4) are given in Figures 3 and 5, and were similar to those for the second tumor, apart from contamination of the S-1 fraction with more thyroglobulin in the latter. The results in Figure 4 were obtained with a third tumor (rat no. 6) which was fractionated only into material which was soluble or insoluble in 1.92 *M* phosphate buffer. Similar results were obtained with the other two tumors.

The salting-out curves (Fig. 3) demonstrate that purified thyroglobulin was completely precipitated at 1.98 *M* phosphate buffer, and that the S-1

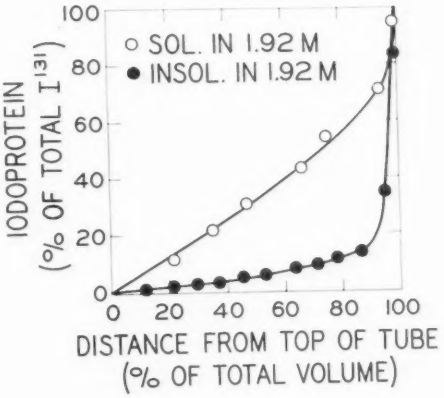

FIG. 4. Sedimentation of soluble iodoproteins from a thyroid tumor in the preparative ultracentrifuge (39,000 rpm, 3 hours, 0.15 *M* NaCl). The iodoproteins were separated according to their solubility in 1.92 *M* KH_2PO_4-K_2HPO_4.

iodoprotein was, indeed, more soluble than thyroglobulin. In the preparative ultracentrifuge (Fig. 4) S-1 iodoprotein was shown to sediment much more slowly than thyroglobulin. The sedimentation rate for S-1 was roughly similar to that found for serum albumin ($S_{20,w}=4.2$) under the same conditions. It is not possible to assign an exact sedimentation coefficient to S-1 from these experiments, however.

Zone electrophoretic analyses (Fig. 5) revealed that the S-1 iodoprotein, unlike thyroglobulin, contained 3 zones: one just behind human serum albumin, one overlapping the alpha globulins and one in the beta globulin area. Reverse flow electrophoresis is illustrated in order to demonstrate that the radioiodine components with the slower mobilities were not artifacts due to adsorption of the fast component on the paper strip. The mobilities were more precisely determined, however, by the conventional zone electrophoresis method. Since the S-1 fraction illustrated in Figure 5 contained very little thyroglobulin (cf. Fig. 3), it appears that S-1 iodoprotein in the rat tumor is composed of at least 3 separate iodoproteins.

The electrophoretic mobility of radioiodine in the thyroglobulin fractions of the rat tumors, and in extracts of tumors, was the same as in normal rat thyroids. In conventional zone electrophoresis this was shown to

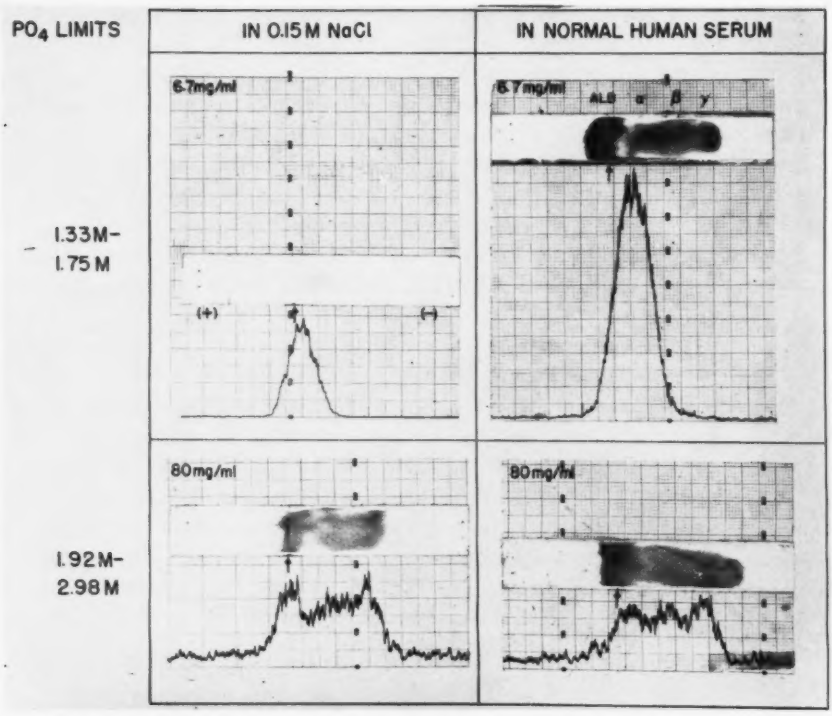

FIG. 5. Zone electrophoresis of soluble iodoproteins from a thyroid tumor (Barbital buffer, pH 8.6 Γ/2 0.1, reverse flow technique). The iodoprotein fractions were isolated by precipitation between the indicated limits of KH_2PO_4-K_2HPO_4, and were dissolved in 0.15 *M* NaCl or normal human serum at the concentrations listed. The paper strips stained with bromphenol blue are mounted above the corresponding records of radio activity. The arrows indicate the points of application. The distance of the base line portions of each record from the zero line indicates the background counting rate.

be intermediate between albumin and alpha-1 globulin of human serum. As pointed out earlier by Alpers, *et al.* (32), rat thyroglobulin has a mobility slightly faster than human thyroglobulin. In several experiments, there was an additional, poorly separated radioiodine peak with the mobility of alpha-1 globulin. This was seen only in conventional zone electrophoresis, and contained 18 percent or less of the total radioiodine. The significance of this finding is uncertain, but could represent yet another iodoprotein.

As in the case of the particulate iodoprotein, the protein nature of the S-1 fraction was revealed by the presence of iodinated amino acids after hydrolysis with pancreatin (Table 3). The results in Table 3 include those obtained on the fractions mentioned above, as well as on a number of other tumors in which the saline extract was divided only into fractions

soluble or insoluble in 1.92 *M* phosphate buffer. Further analysis of the findings after hydrolysis will be discussed below.

In rats no. 4 and 5, the tumor extracts and the fractions obtained from the extracts were examined in the analytical ultracentrifuge. The results are presented in Figure 6 and Table 6. The thyroglobulin fraction (1.4–1.75 *M*) was enriched in a component with $s_{20} = 19$–20 S, but contained much less than did a similar fraction from a normal rat thyroid. This rapidly sedimenting material is similar to thyroglobulin in other species (34). The results of the preparative ultracentrifuge experiments (cf. above)

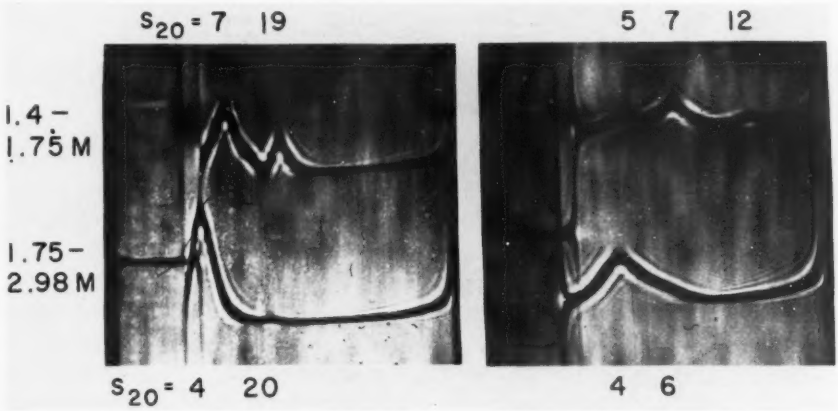

FIG. 6. Sedimentation of soluble protein fractions from a thyroid tumor in the analytical ultracentrifuge (59,900 rpm, 0.4% solution in 0.15 *M* NaCl). The protein fractions were prepared by precipitation between the indicated limits of KH_2PO_4-K_2HPO_4. The values above and below the schlieren diagrams give the sedimentation coefficients, in Svedbergs, of the corresponding boundaries. The photo on the right was taken 28 minutes after the photo on the left. Sedimentation is from left to right. Note the difference in location of the menisci in the standard and "wedge" cells.

indicate that the components of this fraction with sedimentation rates slower than 19S did not contain radioiodine. The S-1 fraction was comprised mainly of slowly sedimenting material which was resolved into two poorly separated components. It is not possible to identify the S-1 iodoprotein with the protein shown in the schlieren pattern, since the proportion of the total protein which is iodoprotein is unknown, but the sedimentation rate for S-1 determined in the preparative ultracentrifuge was of the same order of magnitude.

SERUM IODOPROTEIN

The results of chromatographic analyses of serum radioiodine are presented in Table 7. Although the significance of the small proportion of radioiodine remaining at the origin at the 30-minute point (rat no. 2) may

TABLE 6. DISTRIBUTION OF PROTEINS OF TUMOR EXTRACTS AND A NORMAL RAT THYROID IN THE ANALYTICAL ULTRACENTRIFUGE

Fraction	Rat No.	4–5 S	6–7 S	12 S	19–20 S	27 S
Whole Extract†	5	96*			4	
<1.33M	4	56	39		5	
<1.4M	5	59	32		9	
1.33–1.75M	4	36	46		18	
1.4 –1.75M	5	19	49	5	27	
1.4 –1.75M	Normal		2	8	81	9
1.92–2.98M	4	100				
1.75–2.98M	5	74	22		4	

Values are the per cent of the total area under the schlieren pattern for each peak. Column headings are s_{20} in .15*M* NaCl.

* Partially resolved into 2 peaks with s_{20}=3.9 S and 4.5 S.

† Whole extract of normal rat thyroid studied by Alpers, *et al.* (33) contained 51% of the total area in an 18 S peak; 9% in a faster peak; 6, 7 and 27% in slower peaks.

be questioned, all other samples contained significant quantities of this material. Hydrolysis of this substance in several of the samples (Table 3) revealed it to be iodoprotein. The quantity of radioactive serum iodoprotein as per cent of dose exceeded that of thyroxine at the 3-hour point, but it rose more slowly and decayed more slowly than thyroxine. The peak concentration of iodoprotein was reached at 2 days, whereas that of thyroxine was reached within 1 day.

The iodoprotein in dialyzed serum was characterized by salting-out (4 animals), by ultracentrifugation (1 animal), and by zone electrophoresis (6 animals). The findings in one (rat no. 9) are illustrated and are representative of the group as a whole. In this serum, 69% of the organic radioiodine was iodoprotein and the remainder was in the form of thyroxine. The salting-out of total serum radioiodine is illustrated in Figure 7, together with the salting-out curve after correction for thyroxine-I^{131}. The mean values for solubility of serum thyroxine at each of the salt concentrations (13) were used for this correction. Although there may have been

TABLE 7. SERUM RADIOIODINE IN TUMOR-BEARING RATS

Rat No.	Total I^{131}	Organic I^{131}	Iodoprotein*	Thyroxine	Triiodothyronine
	(% of dose/100 ml.)		(% of organic I^{131})		
2	58	0.58	100		
3	6.4	1.2	80	16	4
4	6.0	3.4	34	66	
5	5.6	4.3	46	54	
6†	9.2	5.2	29	71	
7	6.7	5.4	46	54	
8	7.6	5.3	50	44	6
9	9.0	5.8	69	31	
11	1.6	1.4	76	24	
12	3.3	3.0	78	17	

* Immobile in chromatogram.

† This was the only animal not thyroidectomized prior to study.

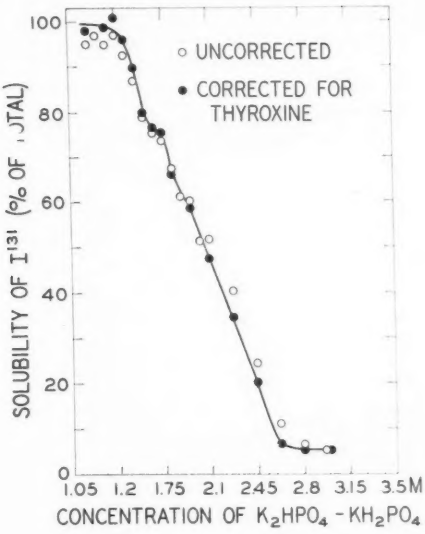

FIG. 7. Salting out of serum radioiodine from a tumor-bearing rat.

a small amount of thyroglobulin (perhaps 20%) present, it is apparent that most of the serum iodoprotein was more soluble than thyroglobulin. The serum iodoprotein, appeared to be slightly less soluble than the tumor S-1 iodoprotein illustrated in Figure 3, but was more like the S-1 fraction in another tumor.

Analysis of serum radioiodine in the preparative ultracentrifuge is shown in Figure 8. The results demonstrate that serum iodoprotein sedimented more slowly than did thyroglobulin in the tumor extract from the same animal.

Zone electrophoresis of serum radioiodine is presented in Figure 9. In these experiments, rat serum was studied alone and in the presence of nor-

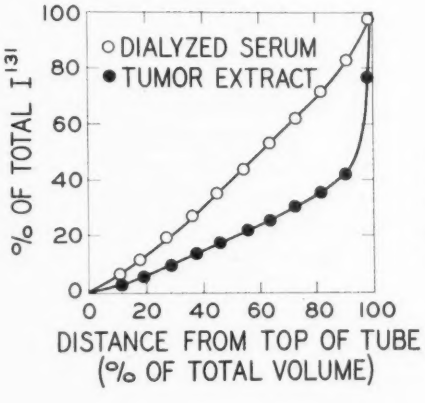

FIG. 8. Sedimentation of radioiodine in dialyzed serum and in the tumor extract from a tumor-bearing rat. (Preparative ultracentrifuge, 39,000 rpm, 120 min.) The serum was diluted 1:34 with 0.15 *M* NaCl. The tumor extract was in 0.15 *M* NaCl.

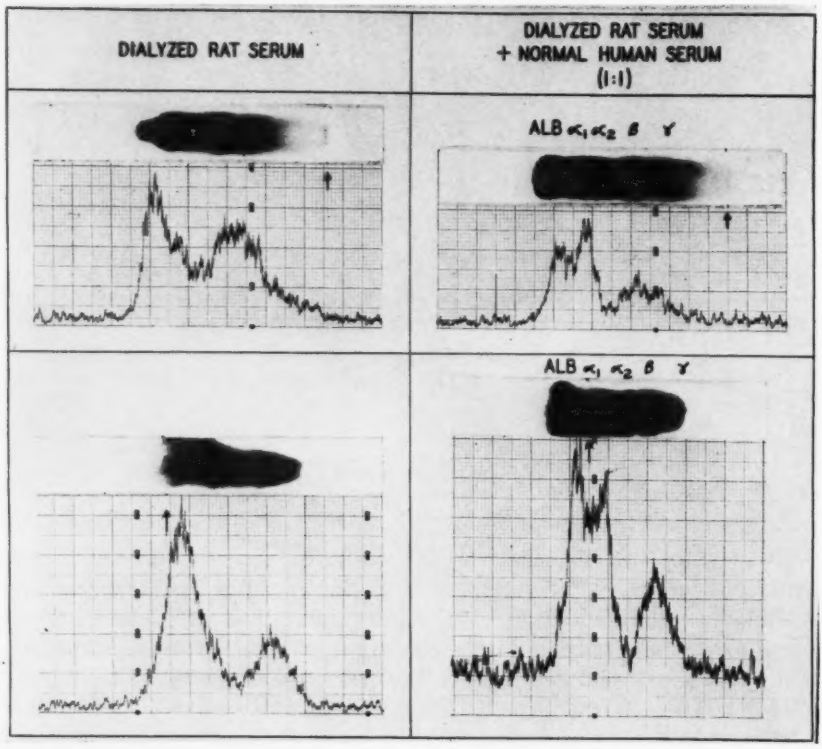

FIG. 9. Zone electrophoresis of radioiodine in dialyzed serum from a tumor-bearing rat. (Barbital buffer, pH 8.6, Γ/2 0.1.) The experiments on the left were performed with rat serum only; those on the right, with a rat serum-human serum mixture. The upper curves were obtained by reverse-flow electrophoresis; those on the bottom, by the conventional method (cf. legend to Fig. 4).

mal human serum. Radiothyroxine was apparently displaced from the fast peak in the rat serum[5] to the alpha globulin in human serum. The other radioactive peaks, then, were due to the serum iodoprotein. Since approximately 34% of the serum radioiodine was found with human alpha globulin in the reverse-flow electrophoresis, and since 31% of the serum radioiodine was thyroxine, it would appear that almost all of the serum iodoprotein was in the radioactivity zones just behind albumin and in the beta-gamma globulin region. This differs from tumor S-1 iodoprotein, where approximately 36% of the *iodoprotein* was in the alpha globulin zone (Fig. 5). The location of the other peaks, however, were similar in the se-

[5] In some runs, the small, rapidly moving protein zone in rat serum was better separated from the other proteins than in Figure 8. Radioiodine was clearly associated with this zone. Although the convention for nomenclature of rat serum proteins is not well established this zone might be designated as prealbumin.

rum iodoprotein and in the tumor S-1 material, and it is possible that not all of the thyroxine shifted to human alpha globulin. Because of this uncertainty, and because the degree of separation of the various radioiodine zones was too poor for precise quantitation, the identity of the serum iodoprotein and tumor S-1 iodoprotein by the criterion of zone electrophoresis remains inconclusive.

HYDROLYSIS OF IODOPROTEINS

As indicated earlier, hydrolysis of the various iodoprotein fractions with pancreatin resulted in the liberation of iodinated amino acids. The results given in Table 3 are the averages for analyses with each of the three chromatographic solvent systems. These analyses agreed fairly closely although it was noted that the butanol-acetic acid system usually gave somewhat higher values for monoiodotyrosine and lower values for diiodotyrosine than did the butanol ammonia system. The values obtained with butanol-dioxane ammonia were closest to the average. Triiodothyronine values were based on the alkaline solvent systems only since triiodothyronine and thyroxine were not resolved by butanol-acetic acid.

In addition to the hydrolytic products identified as monoiodotyrosine, diiodotyrosine, thyroxine, triiodothyronine, and iodide, a number of unidentified products were detected. In some hydrolysates, there were as many as 8 unknown radioactive components. In most instances, these comprised a small proportion of the total radioiodine. One unknown ("U" in Table 3), however, was present in amounts up to 44% of the hydrolyzed radioiodine and was found in approximately one-fifth of the hydrolysates. It had an Rf of approximately 0.6 in the butanol-acetic acid solvent, and approximately 0.8 in both of the alkaline butanol solvents. Comparison of this material with a large number of iodinated tyrosine and thyronine analogues failed to reveal its identity. It was noted, however, that the presence of this material was invariably associated with a decrease in the proportion of monoiodotyrosine, and that the sum of monoiodotyrosine and the unknown gave a value which, in comparison with other hydrolysates, was reasonable to expect for monoiodotyrosine alone. In addition, one iodoprotein fraction which yielded this unknown on hydrolysis, was found on rehydrolysis to contain monoiodotyrosine instead. Since reincubation of hydrolysates containing the unknown with additional pancreatin failed to produce monoiodotyrosine, it seems unlikely that the unknown was a polypeptide containing monoiodotyrosine. It was concluded that this unknown was a complex or derivative of monoiodotyrosine and it was considered equivalent to monoiodotyrosine in analysis of the data.

The hydrolytic products of the particulate iodoprotein differed from those of the soluble iodoproteins in that a larger proportion of the radioiodine (14 to 22%) remained at the origin of the chromatograms. In view of the particulate nature of this material, it was considered that this might

have been the result of inadequate penetration of the enzyme into particles containing iodoprotein. Continued hydrolysis for 48 to 72 hours after the addition of fresh pancreatin, however, produced little, if any, reduction of this component.

Hydrolysates of the tumor S-1 iodoprotein also contained large quantities of radioiodine at the origin of the chromatograms. Measurement of

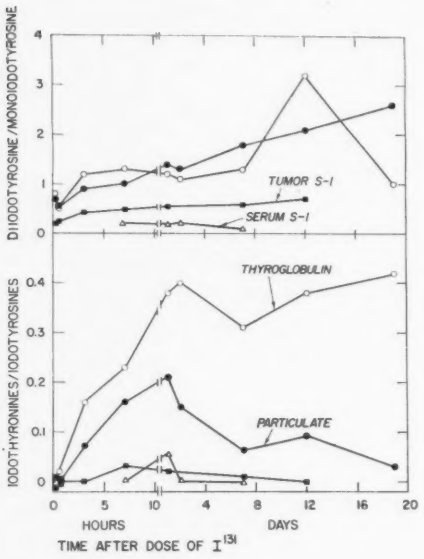

FIG. 10. Comparison of hydrolysates of iodoprotein fractions from thyroid tumors and serum.

the pH at the end of hydrolysis in some of these mixtures indicated that the pH had fallen considerably below the optimum for the pancreatic enzymes.

When the results of hydrolysis of the various fractions were corrected for variations in unhydrolyzed material and iodide (Table 3), it was possible to compare them in terms of the relative quantities of iodinated tyrosines and thyronines liberated by proteolysis. These comparisons are presented graphically in Figure 10. The ratio of diiodotyrosine to monoiodotyrosine, and the ratio of iodothyronines to iodotyrosines in thyroglobulin increased with time after administration of I^{131}. The particulate iodoprotein had the same relative proportions of di- and monoiodotyrosine as did thyroglobulin, but its iodothyronine content was consistently lower. This difference, however, was due only to a lower thyroxine content since triiodothyronine in the particulate iodoprotein was the same as or, perhaps, higher than in thyroglobulin (Table 3). In hydrolysates of the tumor S-1 fraction, diiodotyrosine and the iodothyronines were relatively lower than

in either thyroglobulin or particulate iodoprotein, even though these S-1 fractions were contaminated with thyroglobulin.

The serum iodoprotein subjected to hydrolysis had been separated from other serum radioiodine constituents by chromatography in the butanol-NH_4OH solvent. The origin of the chromatogram was cut out, finely minced, and suspended in the buffered pancreatin. Hydrolysates of the serum iodoprotein contained relatively less diiodotyrosine and iodothyronine than did any of the tumor fractions in the same animals.

In vitro EXPERIMENTS

The results of experiments in which tumor iodine was labeled *in vitro* are presented in Table 8. These data are given separately since they differ

TABLE 8. RADIOIODINE IN THYROID TUMOR SLICES LABELED *In Vitro*

Experiment	Addition	Time of incubation (hours)	Total I^{131} (% of added I^{131})	Organic I^{131} (% of added I^{131})	Particulate iodoprotein* (% of organic I^{131})	Front running component (% of particulate iodoprotein)
1†	0	½	15	2.2	33	46
		3	40	26	23	9
		6	52	40	22	5
2‡	0	3	24	13	28	5
			29	16	28	
	MMI	3	11	0	0	
			12	0.04	100	
3§	0	½	7.0	1.1	42	
			8.0	0.88	40	
	KCl	1	0.79	0.42	50	
			1.4	0.94	30	
		3	3.1	2.6	31	
			3.6	3.1	30	
	KBr	1	2.3	1.0	30	
			2.0	1.1	33	
		3	4.2	3.8	29	3
			3.9	3.5	29	
	KSCN	1	1.6	1.3	33	
			0.71	0.48	42	
		3	0.95	0.85	39	5
			1.2	0.91	34	

* The value for "particulate iodoprotein" includes the front running component (cf. text). Chromatography was not done in those where no value is listed.

† Tissue Slices = 285–290 mg. Medium = 3 ml., 50 μc. iodide—I^{131}.

‡ Tissue Slices = 194–215 mg. Medium = 3 ml., 50 μc. iodide—I^{131}. Addition = 1 methyl-2-mercaptoimidazol (MMI), $10^{-3}M$.

§ Tissue Slices = 140–165 mg. Medium = 3 ml., 50 μc. iodide—I^{131} for first ½ hour. Slices were then removed, rinsed, and transferred to non-radioactive medium containing the addition compound, $10^{-4}M$.

in certain respects from those obtained *in vivo*. The tumor slices functioned well. On a per cent per mg. basis, I^{131} accumulation by the slices was considerably greater than *in vivo*, but such differences obviously depend on tissue: medium ratios on the one hand, and tumor size and tumor blood flow, on the other. Comparison of hydrolysates of soluble iodoproteins in slices incubated 3 hours, with the 3-hour *in vivo* experiment, indicate a lesser iodination *in vitro*. Whereas, the *in vivo* labeled material had a diiodotyrosine:monoiodotyrosine ratio of 3.3 and contained 10% thyroxine, the *in vitro* labeled soluble iodoproteins had a D:M ratio of about 1, and contained 1% or less thyroxine. By 6 hours, the soluble iodoproteins in the slices contained only 5% thyroxine, in contrast to 24% at 7 hours *in vivo*.

A considerable proportion of the radioiodine in the slices was in the form of iodide. It is uncertain, however, whether this was due entirely to inadequate rinsing of the slices prior to analysis, or whether organification of iodine was a rate limiting step. The relative proportion of radioiodide decreased with time.

When calculated on the basis of per cent of organic I^{131} in the slice, the proportion in the particulate fraction was higher than in *in vivo* labeled tumor at similar time intervals. At the ½ hour time, at least, this difference appears to be due to the inclusion of material other than iodoprotein in the particulate fraction. This material moved to the solvent front on chromatography and was found to be soluble in petroleum ether. A similar front running component was inconsistently present in the *in vivo* experiments, and comprised less of the particulate fraction. It may be similar to the front running component found after incubation of thyroid homogenates with radioiodide (35). Another possible explanation for the relatively higher particulate iodoprotein in the *in vitro* experiments is the loss of soluble iodoprotein from the slices to the medium during incubation.

Soluble iodoproteins formed by the slices were similar to those found *in vivo*, but some differences were noted. The relative quantity of S-1 iodoprotein determined by salting-out was the same (Table 4) but the solubility of thyroglobulin in phosphate buffer may have been slightly lower. In zone electrophoresis, the fast peak moved with human alpha-1 globulin, which was slower than for *in vivo* labeled material, and a large proportion of radioiodine was found in a broad peak extending over the remainder of the globulin areas. Reverse flow electrophoresis was not performed, and these studies were done in only one *in vitro* experiment. These results require confirmation, inasmuch as soluble iodoproteins formed *in vivo* and *in vitro* by the same human thyroid tissue (25) were not significantly different.

EFFECT OF ANTITHYROID AGENTS ON IODOPROTEIN FORMATION

Two agents known to interfere with iodination in the thyroid, but acting at different steps, were tested for their effect on the formation of the other iodoproteins. The results are given in Table 8. In Experiment 2, methyl

mercaptoimidazole was shown to prevent the formation of iodoprotein by tissue slices. The small amount present in one is probably of no significance.

In Experiment 3, tissue slices were incubated for ½ hour with radioiodide and then transferred to a non-radioactive medium containing the test substance. In the presence of KCl or KBr, the iodide carried over with the slice was lost to the medium, and then, in part, incorporated into particulate and soluble iodoprotein. KSCN prevented further incorporation of radioiodine. It is of interest that during the 2½ hour interval in the presence of KSCN there appeared to be no change in the relative proportions of particulate and soluble iodoprotein. There was no evidence, therefore, for conversion of particulate to soluble iodoprotein, but the time of the experiment may have been too short or KSCN may have inhibited such a reaction.

COMMENTS

The data presented here demonstrate that iodine-containing proteins other than thyroglobulin can be detected in the tumor and in the blood of rats bearing a transplantable thyroid tumor. The accompanying paper indicates that these findings are not unique for neoplastic thyroid tissue, but can be found in the normal thyroid gland, and also that they are not unique for the rat. The results of hydrolysis with proteolytic enzymes show clearly that these iodoproteins are neither the result of occlusion or addition of inorganic iodine to protein, nor the result of inclusion of thyroglobulin in the various fractions. These iodoproteins have not been isolated in pure form but certain of their physico-chemical properties have been studied by indirect means, employing radioiodine.

Two distinct categories of iodoprotein have been found. One is insoluble in 0.15 M NaCl, and appears to be associated with cell nuclei. The other is soluble in 0.15 *M* NaCl, as is thyroglobulin. It is proposed that these classes of iodoproteins be designated "particulate iodoprotein" or "P," and "soluble iodoprotein," or "S." These may be further identified by prefixing the species and tissue of origin, and suffixing an identifying number. Thus, this paper has described three iodoproteins other than thyroglobulin: rat thyroid P-1, rat thyroid S-1, and rat serum S-1. The S-1 iodoproteins might be further classified by their electrophoretic mobility. Thyroglobulin, which denotes a well characterized protein species, would continue to be called thyroglobulin.

The particulate iodoprotein described in this paper is different from that which has been found after incubation of thyroid tissue homogenates with radioiodine (35). The latter material, when fractionated into subcellular particles, is found largely in the "mitochondrial" fraction (36). Taurog, Potter and Chaikoff (35) found, however, that isolated nuclei from sheep thyroid cells were capable of forming small amounts of iodoprotein *in vitro*. The relationship of this material to the P-1 iodoprotein produced *in vivo* and in tissue slices remains to be determined.

Rerabek (37), on the basis of the sodium nitrite color reaction, described the presence of thyroxine and diiodotyrosine in hydrolysates of cell nuclei isolated from swine thyroids amounting to 2.7% and 4.7%, respectively, of the total quantity of each substance in the glands. Other cell particles contained much smaller quantities. The identification methods employed were less specific than chromatographic analysis and the method for preparing nuclei was different from that used here. Taurog and co-workers (35), on the other hand, found the nuclear fraction of sheep thyroid to contain 0.35 to 2.9% of the total thyroid I^{127}, whereas the mitochondrial-microsomal fraction contained 3.3 to 5.5%.

It is not entirely certain that P-1 iodoprotein is actually part of the cell nucleus. Examination of the nuclear fraction by light microscopy revealed, in addition to a uniform collection of nuclei, an internuclear amorphous material which stained pink with hematoxylin and eosin. Electron microscopy has not been done on these preparations and autoradiography has so far been unsatisfactory.

The finding of iodoprotein in association with a particulate cell fraction suggests the possibility that it might be associated with thyroglobulin synthesis. The change of radioactivity with time in the particulate and thyroglobulin tumor fractions, however, was not as might be expected with such a precursor-product relationship. Instead of an early rise in P-1 followed by a later rise in thyroglobulin, the opposite was found. Lack of specific activity data, however, prevents exact interpretation. The *in vitro* experiment in which KSCN was introduced after labeling of the tumor also failed to demonstrate a conversion of P-1 to thyroglobulin, but the time of incubation may have been too short. The persistently lower thyroxine content of P-1 iodoprotein is also contrary to what might be expected if P-1 was the precursor of thyroglobulin, although it is conceivable that completion of synthesis of the thyroglobulin molecule could precede completion of iodination.

The nature of the P-1 iodoprotein has not been established, but is currently under study.

The S-1 iodoprotein is of interest in relation to the finding of iodoprotein in the blood of patients with certain thyroid disorders. Although they may not be identical, rat serum S-1 and rat thyroid S-1 bear a considerable resemblance to each other. Of further significance is the fact that human thyroid S-1 and human serum S-1, while different from the rat iodoproteins, are, again, similar to each other (14, 25). It seems likely, therefore, that thyroid S-1 iodoprotein is the presursor of the serum iodoprotein. Rat S-1 iodoprotein, unlike that in man, is shown by electrophoresis to consist of several separate components. At the present state of knowledge, however, it seems preferable to refer to them as a unit.

The data presented do not permit any conclusions with respect to the relationship, if any, between S-1 iodoprotein and thyroglobulin. S-1 could be

incompletely synthesized thyroglobulin, partially degraded thyroglobulin or, on the other hand, an alternate pathway for iodination. Since the labeled diiodotyrosine and iodothyronine content of serum S-1 is consistently lower than that of thyroid S-1, it would appear that this substance is secreted at an early stage in its iodination. This is consistent with its early appearance in the blood after radioiodine administration.

The *in vitro* experiments demonstrated that incorporation of iodine by the several iodoproteins was prevented by potassium thiocyanate, which interferes with iodide accumulation, and by mercaptoimidazole, which may act by interfering with iodide oxidation. It remains to be shown, however, whether or not the various thyroid iodoproteins are iodinated by a single iodinating system.

Acknowledgments

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IODOPROTEINS IN NORMAL AND ABNORMAL HUMAN THYROID TISSUE AND IN NORMAL SHEEP THYROID[1]

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ABSTRACT

Two types of iodoproteins other than thyroglobulin have been found in normal and abnormal human thyroid tissue and in normal sheep thyroid. They are similar to, but not identical with, iodoproteins previously described in a functioning rat thyroid tumor, and were detected in tissue labeled with I^{131} either *in vivo* or *in vitro*.

One of these iodoproteins, termed "particulate iodoprotein" or "P-1" was not extractable from thyroid homogenates with 0.15 *M* NaCl. It comprised from 1 to 6% of the organic radioiodine in normal thyroids and in several abnormal thyroid tissues, but was 37% in a follicular carcinoma.

The second iodoprotein, termed "S-1 iodoprotein," was soluble in 0.15 *M* NaCl, but differed from thyroglobulin in other physical properties. It was more soluble than thyroglobulin in phosphate buffer, sedimented more slowly in the ultracentrifuge, and had a faster electrophoretic mobility. It comprised from 1 to 7% of the extractable organic radioiodine in normal and in a wide variety of abnormal thyroids, but was as high as 19% in a follicular thyroid adenoma. Thyroid S-1 iodoprotein resembles iodoprotein previously described in the blood of abnormal human subjects.

One poorly differentiated thyroid carcinoma, which had been labeled *in vitro*, contained iodoprotein which differed from that found in the other tissues.

Thyroglobulin in human and sheep thyroid tissue differed slightly from rat thyroglobulin in solubility in phosphate buffer and in electrophoretic mobility. There was also a small difference in electrophoretic mobility detected between thyroglobulins in the various human tissues.

The manner in which the non-thyroglobulin iodoproteins participate in thyroid physiology is not yet understood.

HITHERTO undescribed iodoproteins discovered in a transplantable thyroid tumor in the rat are described in an accompanying paper (1). This report is concerned with the presence of analogous iodoproteins in human and sheep thyroid tissue. Literature pertinent to this problem is reviewed in the previous paper.

MATERIALS AND METHODS

Tissue was obtained from 16 subjects undergoing surgical thyroidectomy. Normal thyroid tissue was obtained when the thyroid disease was clearly localized or was non-

[1] Presented in part at the 1958 Meeting of the American Goiter Association.

functional, or from subjects undergoing laryngectomy for carcinoma of the larynx. Thyroid glands from freshly killed sheep were pooled.

Details concerning the patients are given in Table 1. The case numbers identify individual patients, in some of whom more than one type of tissue was studied. The tissue diagnosis is based, in each instance, on histological examination. In case 2, the normal thyroid tissue was infiltrated with anaplastic thyroid carcinoma. Analysis of a lymph node metastasis from this tumor indicated that it had minimal function in comparison to the thyroid gland. In case 5, the normal thyroid was the site of metastasis from an adenocarcinoma thought to be of prostatic origin.

Case 7 was clinically euthyroid, although the thyroid gland showed moderate hyperplasia. The tissues listed as "nodular goiter" comprise a heterogeneous group in which a more specific diagnosis could not be made. The thyroid architecture, although for the most part well differentiated, exhibited much pleomorphism in terms of cell height, amount of colloid, shape of the follicles, etc. In cases 9, 10 and 11, portions of the goiter which appeared different macroscopically were analyzed separately. None of these glands had been associated with clinical hyperthyroidism or hypothyroidism.

The tissues listed as "follicular adenoma" were characterized by their occurrence as discrete, encapsulated areas of abnormal tissue in an otherwise normal gland. The fetal adenoma was of microfollicular type and involved almost the entire thyroid gland.

The tissues listed as "carcinoma" were obtained from metastases in the neck which were clearly outside of the thyroid gland except in case 16. In the latter the large, anaplastic tumor contained scattered follicles which may have been normal thyroid tissue. In case 15, the normal thyroid had been ablated when the patient had received 250 mc. of I^{131} for therapy of other metastases 6 years previously. Surgical excision of recurrent tumor was demanded by tracheal obstruction. In case 13, the residual thyroid gland remaining from partial thyroidectomy 23 years previously was removed and found to be abnormal but, apparently, not carcinomatous. It is listed as "nodular goiter." The follicular carcinomas in both case 13 and 15, although predominantly well differentiated, displayed the usual pleomorphism and contained areas of anaplastic epithelium.

Labeling of these tissues was performed *in vivo* by the oral administration of radioiodide, or *in vitro*, as indicated in Table 1. In cases 2, 7 and 12, some of the *in vivo* labeled tissue was also incubated *in vitro* with I^{131}. Since the latter procedure introduced a much higher quantity of radioiodine, it was possible to study both *in vivo* and *in vitro* labeled iodoproteins in the same tissue.

In the *in vitro* procedure, fresh tissue slices approximately 0.2 to 0.5 mm. thick were incubated in an oxygenated Krebs-Ringer phosphate medium, pH 7.4 at 37° C in an oscillating water bath. The duration of incubation is given in Table 1. The tissue slices: medium ratio varied from 150 to 330 mg./ml. Except in cases 3, 7 and 11, however, the quantity of I^{131} accumulated by the slices, as given in Table 1, was measured in separate experiments in which the ratio was 35 to 70 mg./ml.

The *in vivo* radioiodine doses were from 50 to 300 μc.; the time between dose and tissue removal are given in Table 1. The quantity of I^{131} accumulated *in vivo*, as listed in Table 1, was determined by a separate tracer dose given a short time before the experimental dose, and accumulation was measured at 24 hours.

The methods employed for fractionation and analysis of iodine-containing component of the tissues were the same as those described before (1).

RESULTS AND DISCUSSION

The quantity of radioiodine accumulated by the human thyroid tissues, and the proportion of this iodine which was in organic form, are listed in Table 1. The latter figure represents radioiodine which did not move from

TABLE 1. CLINICAL DATA AND RADIOIODINE ACCUMULATION BY HUMAN THYROID TISSUE

Case No.	Thyroid tissue diagnosis*	Age (years)	Sex	Method of labeling	Time of labeling (hours)	Total tissue I^{131} †	Organic I^{131} (% of total tissue I^{131})	Particulate iodoprotein¶ (% of organic I^{131})	Front running component (% of particulate I^{131})	Electrophoretic mobility of thyroglobulin#
1	Normal	53	M	*In vivo*	48	27‡	100			
2	Normal	33	M	*In vivo*	48	28	100			α-1
				In vitro	2.5	19	24			α-1
3	Normal	54	F	*In vitro*	4	5.7	32			slow α-1
4	Normal	30	F	*In vitro*	4	—	83	1.2	4	α-1
5	Normal	71	M	*In vitro*	3	—	78	5.6	9	
6	Normal	60	F	*In vivo*	24	—	100	>4, <7	0	
7	Diffuse Hyperplastic Goiter	27	F	*In vivo*	72	50	100			slow α-1
				In vitro	4	3.2	9			slow α-1
8	Chronic Thyroiditis (Hashimoto's)	50	F	*In vitro*	8	12	25			α-1
9a§	Nodular Goiter*	34	M	*In vitro*	4.5	51	85			slow α-1
9b					4	26	44			slow α-1
10a	Nodular Goiter	45	F	*In vitro*	4	29	78			α-1
10b					4	22	88			α-1
11a	Nodular Goiter	54	F	*In vitro*	4	4.0	71			inter α-1-2
11b					4	—	54			slow α-1
12	Nodular Goiter	37	F	*In vivo*	24	29	100			
				In vitro	3.5	27	92			slow α-1
13 (1)	Nodular Goiter	35	M	*In vitro*	4	—	20	5	3	α-1
1	Follicular Adenoma			*In vivo*	48	‡	100			
3	Follicular Adenoma			*In vitro*	4	5.0	26			inter α-1-2
4	Follicular Adenoma			*In vitro*	4	—	77	4.8	5	slow α-1
14	Fetal Adenoma	19	M	*In vitro*	5	—	5			slow α-1
15	Follicular Carcinoma	66	F	*In vitro*	4	2.4	31			α-1
13 (2)	Follicular Carcinoma			*In vitro*	4	—	12	37	<3	slow α-1
2x‖	Anaplastic Carcinoma			*In vitro*	2.5	2.1	2			
2y					5	2.3	11			
16	Giant and spindle cell Carcinoma	82	F	*In vivo*	144	—	—	5.9		

* Cf. text for further details.
† *In vivo* = % of dose at 24 hours. *In vitro* = % of total I^{131} added/100 mg. tissue.
‡ Proportion of total uptake accumulated by the adenoma is not known.
§ Letters a and b refer to macroscopically different portions of the same tissue.
‖ Letters x and y refer to tissue removed at different times.
¶ The value for particulate iodoprotein includes the front running component (cf. text).
# Given as the serum globulin zone coincident with the radioiodine peak (cf. text).

the origin on chromatography, and was shown by hydrolysis to be composed of iodoproteins (cf. below). Iodide comprised almost all of the remaining radioiodine. In the *in vitro* experiments, it is not known what proportion of this iodide was due to mechanical occlusion only. Smaller quantities of other radioiodine components were sometimes found on the chromatograms, as described in the previous paper (1).

PARTICULATE IODOPROTEIN

In the early phases of this work, the existence of a thyroid radioiodine fraction insoluble in 0.15 *M* NaCl was unsuspected. Consequently, in only 7 of the cases studied was this "particulate" radioiodine measured. In one follicular carcinoma, the value of 37% for particulate iodoprotein was similar to that found in rat thyroid tumors (1). In the other tissues, normal or abnormal, the values were in the vicinity of 6% or less. Such small values might be considered insignificant and due to procedural artifacts. The results of hydrolysis (cf. below), however, revealed differences in iodinated amino acid constituents between the particulate and the soluble iodoproteins in about half of the cases.

Separation of the tissue homogenate into subcellular particulate fractions was performed in case 4, a normal thyroid gland, by means of the two-phase sucrose technique of Hogeboom, Schneider and Striebech (2), employing added $CaCl_2$. The "nuclear" fraction contained 3.8% of the organic iodine in this tissue, which had been labeled *in vivo*. This finding requires confirmation.

Chromatographic analysis of the particulate fraction was performed in all but one instance. Almost all of the radioiodine remained at the origin, 3% or less was iodide, and from 0 to 9% moved with the solvent front (Table 1).

SOLUBLE IODOPROTEINS—HUMAN THYROID TISSUE

Salting-out curves in phosphate buffer (equimolar KH_2PO_4 K_2HPO, pH 6.5) were carried out with the soluble iodoproteins from each of the tissues. In about $\frac{1}{3}$ of these, a 0.15 *M* NaCl extract of the tissue was used as starting material. In the others, the supernatant phase of tissue homogenized in 0.15 *M* NaCl was used. In two instances (case 4—normal, case 11), separate salting-out curves of extract and supernatant were not significantly different. All curves except for that in case 16 were performed with added normal human serum.

A typical salting-out curve is illustrated in Figure 1. Most of the soluble iodoprotein precipitated at low buffer concentrations in a manner typical for thyroglobulin. In Table 2, the buffer concentration at which each salting-out curve exhibited its steepest slope is listed. These values ranged from 1.54 to 1.68 *M* in the normal human thyroids (mean $1.60 \pm .05$),[2] and

[2] Mean ± standard deviation.

from 1.50 to 1.68 *M* in the abnormal tissues (mean $1.57 \pm .05$). The overall mean was $1.58 \pm .05$. In the carcinoma from case 2, a value is not given since the salting-out curve was very much different from all the others. Apparently this tissue did not incorporate I^{131} into thyroglobulin (cf. below). It seems likely that the variations between individual curves were due to experimental error, and that the solubility of thyroglobulin from abnormal tissues was not significantly different from normal. In those experiments in which normal and abnormal tissue were examined in the same subject (cases 1, 3 and 4), the slope maxima agreed closely. Comparison of thyroglobulin labeled *in vivo* and *in vitro* showed no significant difference in case 2-normal and case 12, but the latter appeared to be less soluble in case 7.

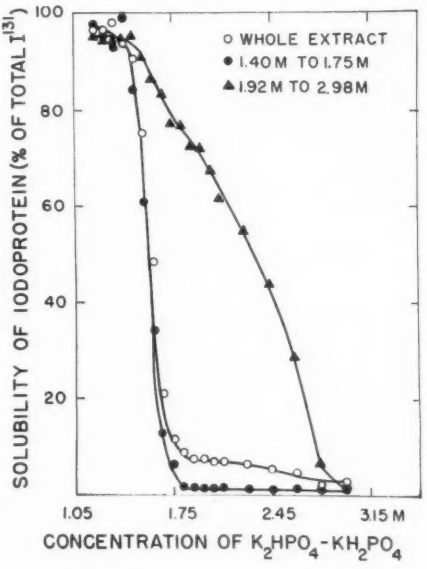

FIG. 1. Salting-out of soluble iodoproteins in the follicular adenoma from case 4. The solid symbols represent iodoprotein fractions isolated by precipitation between the indicated limits of KH_2PO_4-K_2HPO_4.

The salting-out curve of the tissue extract in Figure 1 also demonstrates the presence of a small amount of iodoprotein which is more soluble than thyroglobulin in phosphate buffer. Quantitation of this material is indicated in Table 2, where it is listed as "thyroid S-1 iodoprotein." The values were calculated by subtracting the iodoprotein soluble in 2.98 *M* phosphate buffer from that soluble in 1.89 *M*. In seven instances, salting-out curves were performed on undialyzed extracts or supernatants labeled *in vitro*, whereas all other curves were obtained either with *in vitro* labeled material which had been dialyzed against a large volume of .001 *M* phosphate buffer, or on *in vivo* labeled material. As indicated in Table 2, it was necessary to correct the S-1 iodoprotein values in these seven instances for the iodide

TABLE 2. SALTING-OUT OF SOLUBLE IODOPROTEINS IN HUMAN AND SHEEP THYROID TISSUE

Case No.	1.19 M	1.4 M	1.89 M	2.98 M	S—1	Slope maximum
			Normal human			
1	100	97.0	7.0			1.68
2 (*in vivo*)	99.0	91.0	9.9	5.0	4.9	1.58
2 (*in vitro*)	98.5	84.7	11.1	4.4	6.7	1.54
3	100	92.1	7.6	3.6	4.0	1.54
4	95.7	94.0	2.6	1.5	1.1	1.61
5	96.2	94.6	5.0	2.3	2.7	1.61
6	92.0	88.0	7.1	5.7	1.4	1.61
			Abnormal human			
7 (*in vivo*)	104	97.8	9.6	9.9	0	1.64
7 (*in vitro*)	93.8	82.2	6.6	5.8	0.8	1.50
8	105	88.0	8.8	1.9	6.9	1.50
9a	97.4	89.8	32.1	26.6*	(2.5)	1.58
9b	97.2	91.4	56.2	46.3*	(4.8)	1.50
10a	100	102	46.5	40.0*	(2.1)	1.61
10b	98.2	96.5	47.3	41.5*	(1.2)	1.61
11a	98.9	90.7	33.8	27.2*	(3.6)	1.54
11b	98.4	95.1	78.9	69.1*	(2.1)	1.50
12 (*in vivo*)	98.2	93.7	5.2	3.3	1.9	1.58
12 (*in vitro*)	97.8	93.7	30.7	25.3*	(1.6)	1.58
13(1)	96.6	91.4	5.2	1.4	3.8	1.58
1	101	98.0	11.2	7.7	3.5	1.68
3	97.2	93.4	24.0	4.7	19.3	1.50
4	96.5	93.8	7.6	2.8	4.8	1.61
14	89.0	85.4	18.2	7.6	10.6	1.61
15	100	95.8	8.1	1.3	6.8	1.58
13(2)	94.4	90.6	12.0	8.8	3.2	1.58
2x	79.6	68.0	59.0	41.5		
2y	89.1	75.6	56.4	36.8		
16†	93.2	88.5	9.2	7.4	1.8	1.64
			Normal sheep			
	95.6	80.6	3.6	1.9	1.7	1.45

Columns 2 through 5 give the per cent solubility of radioiodine at the indicated concentrations of KH_2PO_4-K_2HPO_4. Column 6 is the difference between columns 4 and 5. Column 7 gives the molar concentration of KH_2PO_4-K_2PHO_4 at which the salting out curve of thyroglobulin had its maximum slope.

* These preparations were labeled *in vitro* and were not dialyzed prior to salting out. The value for S-1 in parenthesis has been corrected for iodide (cf. text).

† The preparation employed for salting-out contained .877 mg. of lyophylized tissue extract, 8 ml. of 0.15 *M* NaCl, and 16 ml. of water. Serum was not added.

which would salt out over the same range of buffer concentration presumably as a result of interaction with serum albumin. From experiments performed earlier (3), it was known that approximately 10% of iodide I^{131} added to normal serum precipitates between 1.89 and 2.98 *M* phosphate buffer.

In normal thyroids, S-1 iodoprotein comprised from 1.1 to 6.7% of the soluble iodoprotein (mean 3.5%). Most of the values for the abnormal tissues fell within the normal range. In case 7, it may have been lower, and in the adenomas from cases 3 and 14 it was higher than in normal tissue. In the carcinoma from case 2, a value is not given since the entire salting-out curve differed from those obtained with all other tissues (cf. below).

In the normal and adenoma tissue from case 4, the saline extracts were

TABLE 3. FRACTIONATION OF SOLUBLE IODOPROTEINS IN HUMAN AND SHEEP THYROID TISSUE

Thyroid tissue	Phosphate fraction	% of total tissue wt.	% of total extract I^{131}*	Thyroglobulin† (% of I^{131} in fraction)
Normal-Case 4	<1.4 M	0.5	3.6	
	1.4 –1.75 M	3.4	61.	
	1.92–2.98 M	0.3	0.4	32
Adenoma-Case 4	<1.4 M	0.5	8.5	
	1.4 –1.75 M	0.6	49.	
	1.92–2.98 M	0.4	2.5	23
Normal sheep	<1.33 M	0.3	0.6	
	1.4 –1.75 M	7.9	41.	
	1.75–2.98 M	0.2	1.3	24

* The tissue extract was dialyzed prior to fractionation. "Total" refers only to non-dialyzable radioiodine.

† Determined from salting out curve.

fractionated with phosphate buffer as indicated in Table 3. These data should be considered in the light of recovery experiments which showed that when whole extracts of the normal and adenoma tissues were carried through the dialysis and lyophylization steps used in preparation of these fractions, the recovery of radioiodine was 70 and 75%, respectively. From the data in Table 3, it is apparent that the adenoma contained much less thyroglobulin (1.4–1.75 *M* fraction) than did the normal thyroid on a weight basis. The distribution of radioiodine in the two tissues was similar, although there was a higher recovery of I^{131} in the 1.92–2.98 fraction (S-1) in the adenoma in keeping with the findings on salting-out curves prepared from the same tissue extracts (Table 2). As discussed in the previous paper (1), each fraction was prepared by 1 or 2 cuts at the concentrations indicated so that contamination of the S-1 fraction with thyroglobulin was expected.

Comparisons of the thyroglobulin fraction and the fraction more soluble

than thyroglobulin are presented in Figures 1, 2 and 3. The data for the adenoma are shown since there was less contamination of the S-1 iodoprotein with thyroglobulin, but the findings were similar in the normal tissue.

In Figure 1, salting-out curves demonstrate that the 1.92–2.98 *M* fraction, except for apparent contamination with thyroglobulin, was much more soluble than thyroglobulin. The thyroglobulin in the 1.4 to 1.75 *M* fraction, on the other hand, had been freed of S-1 iodoprotein.

In Figure 2, the sedimentation of S-1 iodoprotein in the preparative ultracentrifuge is shown to be much slower than that of thyroglobulin. The small amount of radioiodine in the 1.92–2.98 *M* fraction at the bottom of the tube (13% of the total) is apparently due to contaminating thyroglobulin, and suggest that the amount of contamination estimated from the salting-out curve (Fig. 1, Table 3) may be overestimated. In another ultra-

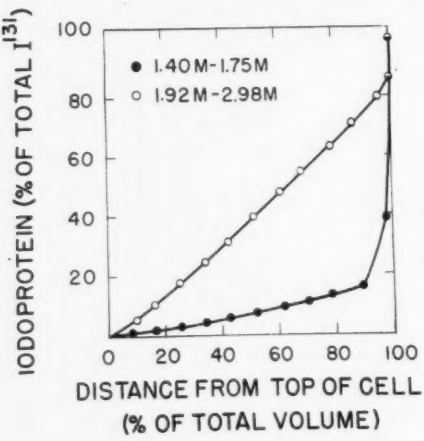

FIG. 2. Sedimentation of soluble iodoprotein fractions (follicular adenoma, case 4) in the preparative ultracentrifuge (39,000 rpm, 3 hours, 0.15 *M* NaCl).

centifuge experiment, fractions prepared from the soluble iodoproteins of case 3—adenoma were compared directly with human serum albumin (Cohn Fraction V). The sedimentation curve of albumin (determined by ultraviolet absorption at 275 mμ) was closely similar to that of iodoprotein which was soluble in 2.28 *M* phosphate buffer.

Zone electrophoretic analyses are shown in Figure 3. Whereas thyroglobulin had a mobility like that of alpha globulin in human serum, most of the radioiodine in the 1.92–2.98 *M* fraction had a mobility like that of serum albumin. The remainder of the radioiodine in this fraction was due, at least in part, to contaminating thyroglobulin but there appeared to be a small quantity in the beta globulin zone as well. The presence of serum did not appear to alter the mobility of these fractions. The results of another experiment in which whole saline homogenates of the normal and adenoma tissue in case 3 was used, are shown in Figure 4. The quantity of S-1 iodoprotein in the adenoma was high enough to be apparent in the un-

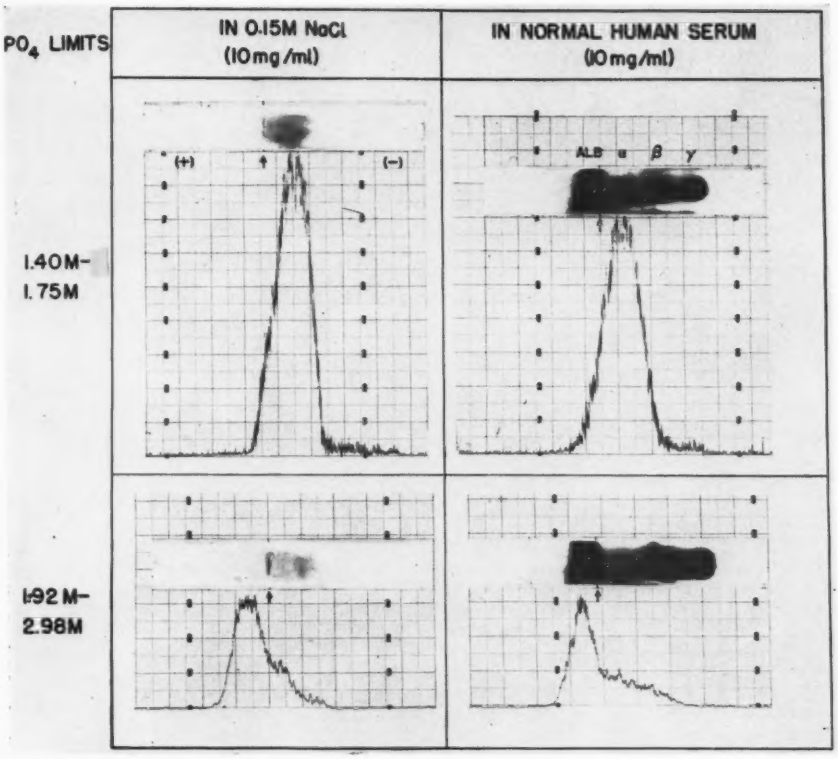

FIG. 3. Zone electrophoresis of soluble iodoprotein fractions from the follicular adenoma of case 4. (Barbital buffer, pH 8.6, $\Gamma/2 = 0.1$, reverse flow technique.) The lyophylized iodoprotein fractions were dissolved in 0.15 *M* NaCl or in normal human serum at the concentrations listed. The paper strips stained with bromphenol blue are mounted above the corresponding records of radioactivity. The arrows indicate the points of application. The distance of the baseline portions of each record from the zero line indicates the background counting rate.

fractionated material as a radioiodine band with the mobility of albumin. The normal tissue, on the other hand, showed only a thyroglobulin zone. The amount of radioiodine remaining at the origin appeared to be due to insoluble, or particulate, radioiodine in the tissue homogenates.

The electrophoretic mobility of thyroglobulin was examined in 12 subjects. For these analyses, a small quantity of tissue homogenate, supernatant, or extract was added to normal human serum, and the mixture was separated by conventional zone electrophoresis. A typical result is shown by the normal thyroid in Figure 4. The major radioiodine band extended, in all cases, from the anodal end of the beta globulin zone to the anodal end of the albumin zone. The peak of the radioiodine zone varied slightly, as indicated in Table 1, and was either coincident with the alpha-1 globulin

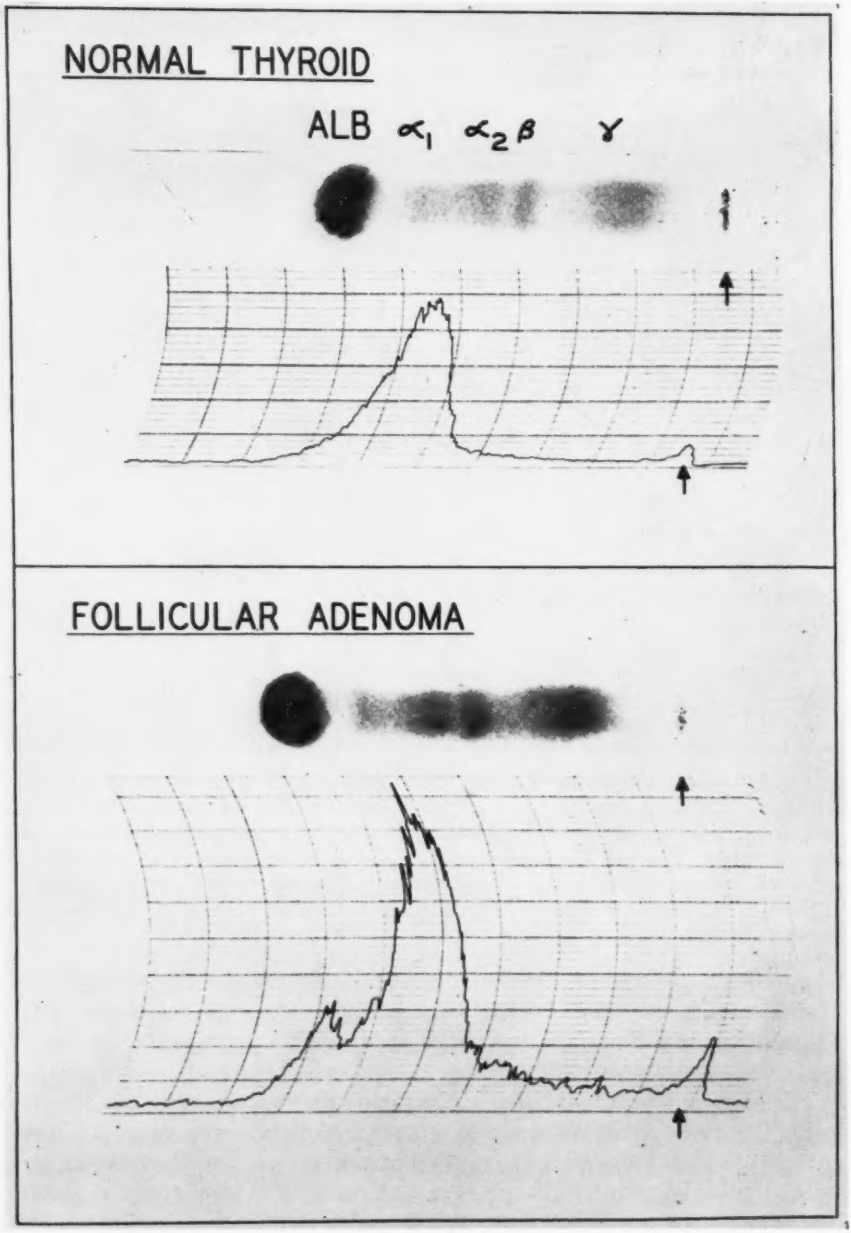

FIG. 4. Zone electrophoresis of unfractionated soluble iodoproteins in the normal thyroid and in the follicular adenoma of case 3. (Barbital buffer, pH 8.6, $\Gamma/2$ 0.1, conventional technique.) The anode is at the left. Cf. Figure 3.

zone or with the inter-alpha globulin space, or was intermediate between these. The latter is listed in Table 1 as "slow α-1." No consistent difference was found between the normal and abnormal tissue groups, but in normal and abnormal tissue from the same subject, minor differences were sometimes seen (cf. Fig. 4). There was no difference between thyroglobulin labeled *in vivo* and *in vitro*. In about one-half of these experiments, sufficient thyroglobulin was present to be visible on the stained protein strip. It appeared to extend from the inter-alpha space to the anodal end of the alpha-1 zone, and sometimes obscured the alpha-globulin landmarks.

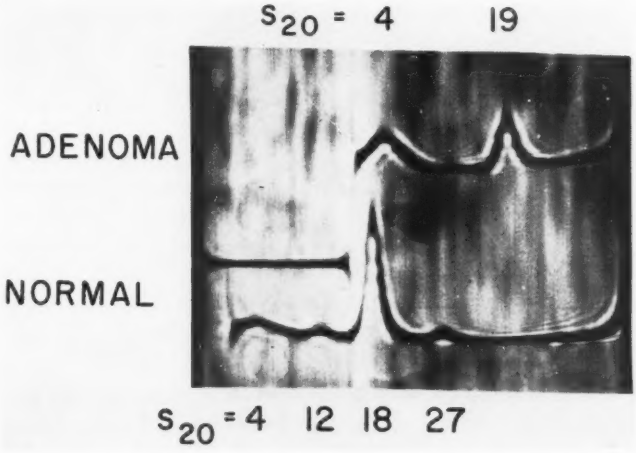

FIG. 5. Sedimentation of the unfractionated saline extracts of the normal thyroid and follicular adenoma of case 4 (59,900 rpm, 0.4% solution in 0.15 *M* NaCl). The extracts had been dialyzed, lyophylized and redissolved in the saline. The values above and below the schlieren patterns give the sedimentation coefficients, in Svedbergs, of the corresponding boundaries. Note the difference in location of the menisci in the standard and "wedge" cells.

Analytical ultracentrifuge analyses of the saline extracts of both normal and adenoma from case 4, and the fractions prepared from these extracts, are presented in Figures 5 and 6 and summarized in Table 4. In keeping with the data in Table 3, the whole extract from the adenoma contained relatively less thyroglobulin ($s_{20} = 19$ S) than did that from the normal tissue. The 1.4–1.75 *M* and the 1.92–2.98 *M* fractions, however, were quite similar in the two tissues. The slow component in each of the more soluble fractions did not break up during centrifugation for 60 minutes but its asymmetry indicated heterogeneity. As discussed elsewhere (1), an identity between the slow component in the schlieren pattern and the S-1 iodoprotein has not been established.

The fractions less soluble than thyroglobulin (Table 3) were not exam-

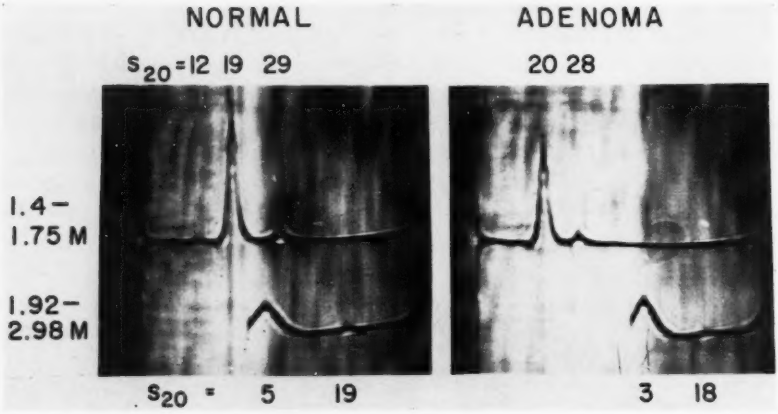

FIG. 6. Sedimentation of soluble iodoprotein fractions (normal thyroid and follicular adenoma, case 4 in the analytical ultracentrifuge (59,900 rpm, 0.4% solution in 0.15 *M* NaCl). The protein fractions were prepared between the indicated limits of KH_2PO_4-K_2HPO_4. Cf. Figure 5.

ined in the analytical ultracentrifuge. As in the rat tumor extracts (1), this material could not be completely redissolved in 0.15 *M* NaCl after lyophilization and may have contained denatured thyroglobulin. The radioiodine in the soluble portion of these fractions resembled thyroglobulin by the criteria of salting-out, sedimentation in the preparative ultracentrifuge, and electrophoretic mobility.

The findings on the carcinomatous tissue from case 2 deserve special comment. The tumor was obtained from cervical lymph nodes, well removed from the thyroid gland, on two separate occasions. Although the tumor contained, for the most part, solid sheets and cords of epithelial cells, sections from the tissue labeled "x" in Table 1 contained some small acinar structures, and those from specimen "y" contained some poorly formed follicles which enclosed eosinophilic material resembling thyroid colloid. The formation of iodoprotein by the tumor slices was unequivocal. In both instances, chromatograms of the tumor homogenates contained more radioiodine at the origin than did chromatograms of the incubation

TABLE 4. DISTRIBUTION OF PROTEINS IN THE SCHLIEREN PATTERNS OF FIGURES 5 AND 6 (PER CENT OF TOTAL AREA UNDER THE CURVES)

Fraction	Tissue	3–5 S	12 S	18–19 S	28–29 S
Whole extract	Normal	12	3	82	3
	Adenoma	50		50	
1.4–1.75 M	Normal		<3	94	6
	Adenoma			88	12
1.9–2.98 M	Normal	87		13	
	Adenoma	93		7	

media. In the case of specimen "y," slices of the patient's skeletal muscle were incubated with I^{131} as a control. Chromatography of the homogenized muscle slices revealed only a trace amount of radioiodine at the origin. Hydrolysis of the tumor homogenates with pancreatin resulted in the liberation of monoiodotyrosine and 2 unknown compounds.

Zone electrophoretic analyses of the homogenate from each tumor, when added to human serum, revealed that from 40 to 53% of the radioiodine remained immobile and, presumably, was associated with particulate material. The remainder of the radioiodine was distributed in a broad smear extending from gamma globulin to albumin, but with some peaking in the alpha globulin region. These findings were complicated by the fact that undialyzed homogenates were used, but most, if not all, of the iodide had moved off the paper strip during electrophoresis.

Salting-out curves were performed on the supernatant portion of these homogenates after exhaustive dialysis against distilled water. As indicated in Table 2, about 40% of the radioiodine remained in solution in 2.98 *M* phosphate buffer. The configuration of the salting-out curves indicated that little, if any, thyroglobulin or S-1 iodoprotein was present. Hence this case synthesized yet another iodinated protein of unusual solubility.

SOLUBLE IODOPROTEINS—SHEEP THYROID

Soluble iodoproteins were also studied in normal sheep thyroid which had been labeled *in vitro*. The salting-out curve of the saline extract, as indicated in Table 2, was similar to that in the human thyroids except for a slightly lower solubility of sheep thyroglobulin. This extract was fractionated with phosphate buffer, as indicated in Table 3. It contained more thyroglobulin on a weight basis than did the extract from the human gland. The thyroglobulin and S-1 iodoprotein fractions gave results entirely analogous to those in the normal human thyroid when studied by salting-out, and sedimentation in the preparative and analytical ultracentrifuges. The results of zone electrophoresis were also similar, but not identical with the human. The radioiodine in the thyroglobulin fraction extended from the cathodal end of the albumin zone to the anodal portion of the beta globulin zone of human serum. Its peak was in the inter-alpha globulin space. In the sheep S-1 iodoprotein fraction, radioiodine was distributed in 2 major zones: 35% in a sharp band in the fast portion of the human albumin zone, 51% in a broad band overlapping the entire alpha globulin area with its peak in the inter-alpha globulin space. The alpha globulin radioiodine was greater than could be accounted for by contaminating thyroglobulin (cf. Table 3).

HYDROLYSIS OF IODOPROTEINS

The results of hydrolysis of the iodoproteins with crude pancreatic enzymes are given in Table 5. Only those experiments are listed in which it

was possible to compare the various fractions. Each of the hydrolysates contained iodinated tyrosines, thus attesting to the protein nature of the materials under study. The low yields of iodinated thyronines was expected since all but one of the tissues were labeled *in vitro*. Not only does *in vitro* labeling appear to be a relatively slow process, but it is furthermore limited to 4 or 5 hours. Most of the hydrolysates contained unidentified radioactive components, as was the case in the rat (1). One of these is listed in the Table, and its relationship to monoiodotyrosine has been discussed elsewhere (1).

Comparison of the hydrolysates of particulate iodoprotein with iodo-

TABLE 5. HYDROLYSIS OF IODOPROTEINS IN HUMAN AND SHEEP THYROID TISSUE

Case No.	O	I	M	D	T_4	D/M	T_4+T / D+M	P	I	M	D	T_4	U	D/M	T_4+T_3 / M
	A. Soluble in 0.15 M NaCl							*B. Particulate*							
Normal human															
4	4	3	64	32	2	.50	.021	10	9	61	28	2		.46	.022
5	8	7	48	40	4	.83	.045	4	16	59	12			.20	0
6	5	7	30	58	11	1.9	.12	33	18	6	69		25	2.2	0
Abnormal human															
13(1)	2	5	60	32	2	.53	.022	10	14	61	30			.49	0
4	4	4	55	36	5	.65	.055	8	8	60	27	5		.45	.058
13(2)	2	10	81	16		.16	0	5	17	95				0	0
	C. Insoluble in 1.92 M phosphate							*D. Soluble in 1.92 M phosphate*							
Normal human															
4	8	4	64	31	3	.48	.031	8	9	81	16			.20	0
5	6	3	55	42	2	.76	.021	2	0	39	16		39	.21	0
Abnormal human															
4	3	5	52	34	9*	.65	.10	9	7	75	18	4		.24	.043
Normal sheep	16	15	65	22	3	.34	.015	10	60	50	17			.34	0

Definition of symbols: O = I^{131} at chromatogram origin, I = iodide, M = monoiodotyrosine, D = diiodotyrosine T_4 = thyroxine, T_3 = triiodothyronine, U = unknown (cf text). Values for O and I are in terms of per cent of total iodoprotein. Values for M, D, T_4 and U are in terms of per cent of hydrolytic products other than iodide and immobile material. Deviation of the sum of these values from 100% is due to other unknowns.

Part A = whole thyroid extract or supernatant. Parts C and D = fractions obtained from the extract or supernatant. In all but case 6, the extract or supernatant was dialyzed prior to hydrolysis.

* This hydrolysate also contained 2% T_3.

proteins soluble in 0.15 *M* NaCl reveals certain differences. The particulate iodoprotein gave rise to relatively more iodide and to more immobile radioiodine. The latter may be unhydrolyzed or partially hydrolyzed protein. Except in one case, this component was not as large as in the rat tumors (1). The origin of the iodide is unclear. Of the remaining hydrolytic products, the relative proportions of diiodotyrosine (D) and monoiodotyrosine (M) were similar in 3 of the hydrolysate pairs. In the other 3, the ratio D/M was lower in the particulate iodoprotein. The low yield of thyroxine and triiodothyronine made the iodothyronine/iodotyrosine ratio somewhat unreliable. In case 6, however, the thyroxine content of particulate radioiodine was clearly lower.

Comparison of thyroglobulin and the S-1 iodoprotein (parts C and D of Table 5) reveals a consistently lower diiodotyrosine and thyronine content

in the latter except in the sheep thyroid. The reason for the unusually large amount of iodide in sheep thyroid S-1 is unknown.

As discussed previously (1) the difference in hydrolysis products among the iodoproteins indicate that the proteins are not artifacts of the fractionation procedures.

COMMENTS

The iodoproteins of human and sheep thyroid tissue described in this paper are analogous to those found in a functioning rat thyroid tumor (1). As proposed in the preceding paper, they may be classified according to their solubility in 0.15 *M* NaCl as particulate iodoprotein, or "thyroid P-1" and soluble iodoproteins, or "thyroid S-1" and thyroglobulin.

The association of human thyroid P-1 iodoprotein with specific subcellular particulate fractions has not been as well delineated as it has in the rat. Even in the rat, however, its apparent association with cell nuclei has not been conclusively proved. The quantity of thyroid P-1 iodoprotein was highest in a follicular thyroid carcinoma, but was apparently present in normal thyroids and in benign goiters as well. Particulate iodoprotein was not studied in the sheep.

The soluble thyroid iodoproteins of man and sheep were different in certain respects from those found in the rat. The solubility of human thyroglobulin in phosphate buffer was slightly greater than that of rat thyroglobulin, and sheep thyroglobulin appeared to be less soluble than either human or rat. These thyroglobulins also differed in electrophoretic mobility, those of man and sheep being slower than rat thyroglobulin. The slight differences in electrophoretic mobility of human thyroglobulin among the tissues studied are like that described in one case by Easty, Slater and Stanley (4).

Thyroid S-1 iodoproteins were different in each of the species studied, as revealed by zone electrophoresis. In man, S-1 iodoprotein had the mobility of serum albumin, although there may have been a small amount moving with the serum globulin as well. In the sheep, S-1 iodoprotein appeared to consist of 2 main components, one with the mobility of human albumin, and one which moved with human alpha globulin. In the rat, S-1 iodoprotein was composed of 3 more or less equal components, the fastest of which moved behind human serum albumin.

It is of considerable interest that human thyroid S-1 iodoprotein bears a close resemblance to the iodoprotein found in the blood of patients with functional carcinoma (5, 6) as well as that in a patient with congenital goiter (7) and, although the data are less complete, in chronic thyroiditis (8). The data reported here demonstrate that thyroid S-1 iodoprotein is present in normal thyroid glands, as well as in tissue representing a variety of benign and malignant thyroid disorders. The highest proportion of this material was found in 2 follicular adenomas. It has not been shown, how-

ever, whether this is the result of an absolute increase in the concentration of S-1 iodoprotein, or a relative decrease in thyroglobulin.

Additional evidence that thyroid S-1 iodoprotein is related to the iodoprotein in blood is the finding that rat thyroid S-1, while differing from thyroid S-1 in other species, resembles the iodoprotein found in the blood of rats with thyroid tumors (1).

Further speculations on the nature of the non-thyroglobulin iodoproteins and their possible significance are discussed in the preceding paper.

Acknowledgments

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THE EFFECTS OF HYPOTHALAMIC LESIONS ON GOITROGENESIS AND PITUITARY TSH SECRETION IN THE PROPYLTHIOURACIL-TREATED GUINEA PIG[1]

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ABSTRACT

Propylthiouracil (PTU)-induced goitrogenesis was selectively impaired in male guinea pigs by anterior hypothalamic lesions. These lesions also reduced or abolished the PTU-induced enlargement of the adenohypophysis and the increase of thyrotrophin (TSH) stores therein, without causing gonad atrophy. More posterior lesions affected TSH secretion less and did cause gonad atrophy. The findings are interpreted to mean that neither the synthesis nor the release of TSH can reach maximal levels in the absence of the neural structures which were damaged.

IN RATS, appropriate hypothalamic lesions have been found to impair, but not abolish, thyrotrophin (TSH) secretion (1–5). In dogs, on the other hand, similar lesions seem to eliminate TSH secretion, or at least its release phase, since they reduce both thyroid size and I^{131} uptake to hypophysectomy-like levels (6). This apparent species difference in the extent to which TSH secretion may be decreased by hypothalamic damage suggests that further comparative studies, such as the one presented here, may be of value. In this investigation the question of whether not only the *release* but also the *synthesis* of TSH can be impaired by lesions of the hypothalamus has been approached through the study of pituitary TSH stores and thyroid activity in the propylthiouracil (PTU)-treated guinea pig. The choice of experimental animal was governed by the following considerations.

The amount of hormone synthesized during an experimental period must equal the algebraic sum of the amounts stored in, and released from, the adenohypophysis. The stored TSH may be assayed directly, but indices of released TSH, such as various thyroid responses or even measurements of circulating TSH levels, are only indirect since they give no indication of how much released TSH may have been cleared or destroyed by extrathyroid mechanisms. In the rat, the effects of hypothalamic lesions are made most evident by chronic PTU-treatment (1–3, 5). However, the pituitary TSH stores of PTU-treated rats are low, despite a rapid rate of TSH synthesis (7). Because of the uncertainty with which release can be

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estimated, the effects of hypothalamic lesions on blood and pituitary TSH levels in such animals (5) may be difficult to interpret. In the guinea pig, on the other hand, chronic PTU-treatment leads to an increase of pituitary TSH stores as well as thyroid enlargement (7, 8). Thus, all 3 phases of TSH secretion—synthesis, storage and release—proceed at accelerated rates during goitrogenesis in this species. In such a situation, the impairment of TSH release by hypothalamic lesions should effect a decrease of thyroid hypertrophy, whereas a lessened capacity for TSH synthesis should tend to diminish adenohypophyseal TSH accumulation.

MATERIALS AND METHODS

Hypothalamic lesions were made, under ether anesthesia, in young (212–370 gm.) and adult (400–650 gm.) male guinea pigs, using a "homemade" stereotaxic device.[2] A direct (anodal) current of 3 to 4½ m.a., applied for 20–45 seconds, was used for each lesion. Coordinates were determined empirically. In each experimental animal 2 or 3 lesions were placed so as to fuse across the midline. Most lesions were made in the anterior hypothalamus, but a few were placed in the median eminence region and some in the anterior thalmus. Sham operations consisted of trephining the skull, with or without electrode insertion.

Postoperatively, the guinea pigs were placed in either wire mesh cages (6–8 young) or wooden pens (7–12 adult). Since no behavioral effects of the lesions were noted, experimental and control animals shared the same quarters. The basic diet was Purina fox chow meal, mixed with PTU[3] (0.15%). Raw vegetable supplements were fed 4–7 times weekly, and tap water was allowed *ad libitum*. Flaked oats, ascorbic acid (2%) and sulfadiazene (2%) with $NaHCO_3$ (6%) were added to the basic diet of the adults (Groups VII and VIII). The sulfadiazene was found to decrease the incidence of respiratory infections. Both young and adult "euthyroid" controls (unoperated) received similar treatment, but without either PTU or sulfadiazene-bicarbonate.

The guinea pigs were fed the PTU diet for 68–113 days, and weighed biweekly. At sacrifice, thyroid-serum radioiodide concentration ratios (T/S) were measured as follows. One hour after a subcutaneous injection of 30 mg. of PTU (in alkaline solution), 20 μc. of NaI^{131} were injected subcutaneously into each animal. Blood samples were removed 60–80 minutes later under ether anesthesia. The guinea pigs were then killed and the pituitary and thyroid glands removed immediately. The radioactivity in 50 mg. of serum and in about 10 mg. of thyroid tissue was measured by β-counting. T/S ratios were calculated conventionally (9).

Adenohypophyses, stripped of neural lobes, were weighed rapidly and frozen (in 0.5 ml. of 0.25% acetic acid-0.85% NaCl solution) for subsequent TSH assay. Brains were

[2] The stereotaxic device utilized a cat-monkey headholder. The ear bars were $2\frac{5}{8}$″ high, their support posts were 3″ apart and the tooth clamp was adjustable. (This headholder, mounted on a 7″ square base, was supplied by the late Mr. Roy Johnson.) A raised (2″) level platform (5″×2″) was mounted on the base plate to the left of the headholder, and the electrode-carrier unit of a Kreig-Johnson rat stereotaxic instrument bolted thereon with #210 thumb bolts. This improvised device allowed the same stereotaxic instrument to be rapidly converted for use on either rats or guinea pigs. A unipolar electrode, protruding 15–17 mm., with 1 to 1½ mm. bare at the tip, was used for the guinea pigs.

[3] The PTU was generously donated by Dr. R. J. Turner of the American Cyanamid Company.

fixed in 10% neutral formalin, Tissuemat-embedded, and sectioned serially at 10μ. Every 9th and 10th section was stained with thionine for lesion-localization studies. Thyroid, testis and seminal vesicle weights were recorded and the tissues processed for histologic study. The organ weights were converted to relative weights (mg./100 gm. body weight at sacrifice) and are discussed as such in this paper.

TSH bioassays were carried out in stasis tadpoles on homogenates of 5–10 pooled anterior lobes, as previously described (10).

RESULTS

Goitrogenesis. PTU treatment for 68–75 days increased the mean thyroid weight of 12 young guinea pigs without hypothalamic lesions approximately four-fold (Table 1, Group II vs. Group I, $P \neq .004$). The same treatment raised thyroid weight less than two-fold in 15 animals with such lesions

TABLE 1. BODY AND ORGAN WEIGHTS AND T/S RATIOS IN "EUTHYROID" CONTROLS AND PROPYLTHIOURACIL-TREATED GUINEA PIGS, WITH AND WITHOUT HYPOTHALAMIC LESIONS

	Group	Body weight (gm.)	Days on PTU	Wt. gain* (gm./day)	Hypophysis†	Organ weights—mg./100 gm.		Thyroid	T/S
						Testis	Seminal vesicles		
I	Euthyroid Control (13)‡	584 ± 15§	0	—	1.70 ± .09	527 ± 28	119 ± 6	9.6 ± 0.7	57 ± 8
II	PTU Control (12)	461 ± 17	68 to 75	1.88 ± .30	2.48 ± .18	550 ± 25	112 ± 9	43.9 ± 10.4	317 ± 72
III	Hypothal. Lesions (15)	444 ± 29		1.86 ± .31	1.99 ± .11	594 ± 27	178 ± 12	17.5 ± 1.8	369 ± 93‖
IV	PTU Control (5)	553 ± 33	102 to 113	2.30 ± .10	3.04 ± .38	537 ± 18	164 ± 27	96.4 ± 9.8	198 ± 5
V	"Goiter Block" (6)	597 ± 49		2.30 ± .42	1.65 ± .18	585 ± 39	176 ± 29	19.0 ± 2.5	271 ± 37
VI	"Ineffective" Lesions (6)	512 ± 14		2.03 ± .23	1.95 ± .27	429 ± 39	122 ± 25	74 ± 19.4	250 ± 45
VII	PTU Control (5)	1071 ± 80	103	2.80 ± .52	1.70 ± .26	355 ± 18	149 ± 17	43.7 ± 5.5	486 ± 54
VIII	"Ineffective" Lesions (6)	938 ± 95		2.35 ± .68	1.26 ± .11	460 ± 35	187 ± 7	39.7 ± 6.7	581 ± 133

* During PTU feeding.
† Anterior lobe only.
‡ No. of animals.
§ S.E. of mean.
‖ Average of only 11 data.

(Group III vs. Group I, $P \neq .001$). The lesions thus decreased goitrogenesis by more than 50% (Group III vs. Group II, $P \neq .02$). However, since some of the PTU-controls did not show appreciable thyroid enlargement, it was not possible to attribute the absence of goiter in individual animals of Group III to their lesions, even though "goiter block" was evident in the group as a whole. To raise control thyroid weights further, so that individual goiter-blocking lesions might be selected, a longer PTU-treatment period (>100 days) was used subsequently. For 5 young PTU-controls (Group IV), the longer treatment resulted in striking (ten-fold, $P < .001$) thyroid enlargement. Similar treatment produced less marked, but still significant ($P \neq .001$), goiter formation in 5 older controls (Group VII). Fifteen younger (Groups V and VI) and six older (Group VIII) guinea

pigs with hypothalamic lesions survived the prolonged PTU regimen. Of the younger, 3 which exhibited severe gonadal atrophy were not included in the table (but are included in Fig. 1); the remainder were divided into "goiter block" (V) and "ineffective lesion" (VI) groups on the basis of thyroid weight. Group VIII did not exhibit "goiter block," although one of its members had a thyroid weight in the "goiter block" range (21.2 mg./100 gm.).

T/S ratio. The average "euthyroid" T/S ratio was 57 ±8. PTU treatment increased this value from four- to ten-fold (Table 1). Neither in Group V nor in other groups did lesions prevent this increase consistently, although a few animals in Group III did have T/S ratios in the "euthyroid" range. The findings even suggest that the lesions tended to increase the T/S ratios, but the average increases were not statistically significant. Thyroid size and T/S ratios tended to be related inversely (Fig. 1).

Thyroid microscopic structure. PTU-treatment for 68–75 days induced

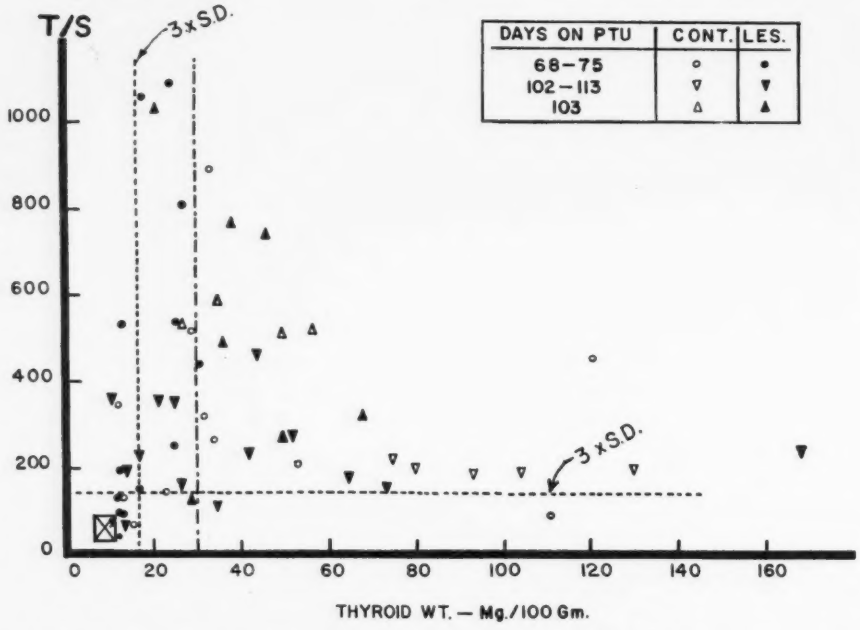

FIG. 1. Graph of thyroid weight and T/S ratio for individual male guinea pigs fed propylthiouracil (PTU) for 68–113 days. The small square with crossed diagonals at the lower left of the graph indicates the mean thyroid weight and T/S ratio (center of square) and standard deviation (perimeter of square) for 13 "euthyroid" controls (Group I, Table 1). The dotted lines which enclose this square represent 3 times the standard deviation and should limit over 99.9% of individual "euthyroid" values. The vertical line made of dots and dashes indicates the arbitrary limit (30 mg./100 g.), above which "goiter block" was not considered to be appreciable. Open symbols = PTU controls (see legend); solid symbols = hypothalamic lesions.

marked changes in the microscopic thyroid structure of controls (Group II), which roughly paralleled in extent the increases of thyroid weight. "Euthyroid" (Group I) glands were characterized by squamous acinar epithelium and dense colloid containing only small peripheral vacuoles (Fig. 2). Three (25%) of the Group II glands, weighing between 12 and 16 mg./100 gm., closely resembled those of Group I; the others had cuboidal or columnar epithelium and whatever colloid they contained was more dilute. In 9 (60%) of the Group III guinea pigs thyroid structure appeared inactive. In general, as thyroid weight increased, cell height did too and

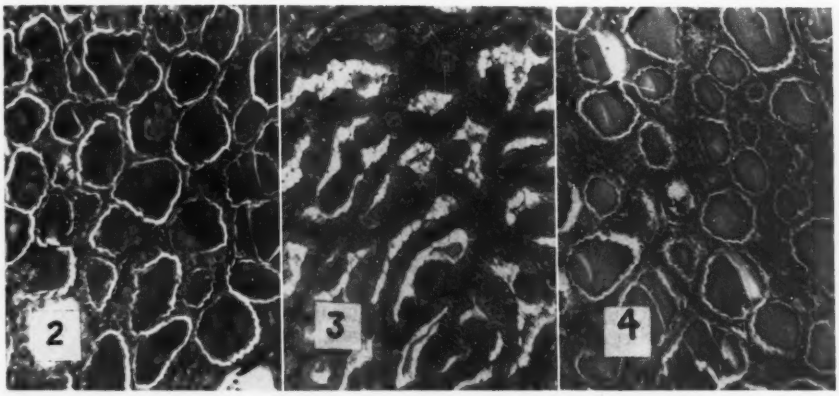

FIG. 2. Thyroid gland of guinea pig not given PTU. Squamous acinar epithelium, dense colloid.

FIG. 3. Thyroid gland of guinea pig fed PTU for 113 days. Weight: 104 mg./100 gm.

FIG. 4. Thyroid gland of guinea pig with "goiter block" after 113 days of PTU treatment. Weight 11.4 mg./100 gm.

the colloid became progressively more dilute and highly vacuolated. The largest glands were virtually devoid of colloid and their follicles appeared collapsed.

The thyroid glands of 102–113 day PTU controls (Group IV) similarly looked highly stimulated (Fig. 3). In Group V, only the largest gland exhibited cuboidal epithelium and dilute colloid. The others had squamous epithelium and colloid which seemed to be only a bit less dense (Fig. 4) than that seen in Group I.

The two smallest glands in Group VI (42 and 52 mg./100 gm.) appeared less stimulated than the rest which resembled those of group IV. However, even these two glands had low columnar epithelium, definitely greater in height than any in Group V.

Pituitary size and TSH content. In the younger controls, PTU treatment for 102–113 days increased anterior pituitary weight 46% (Group IV vs. Group I, $P = .001$). Both Group V and Group VI lesions reduced this hyper-

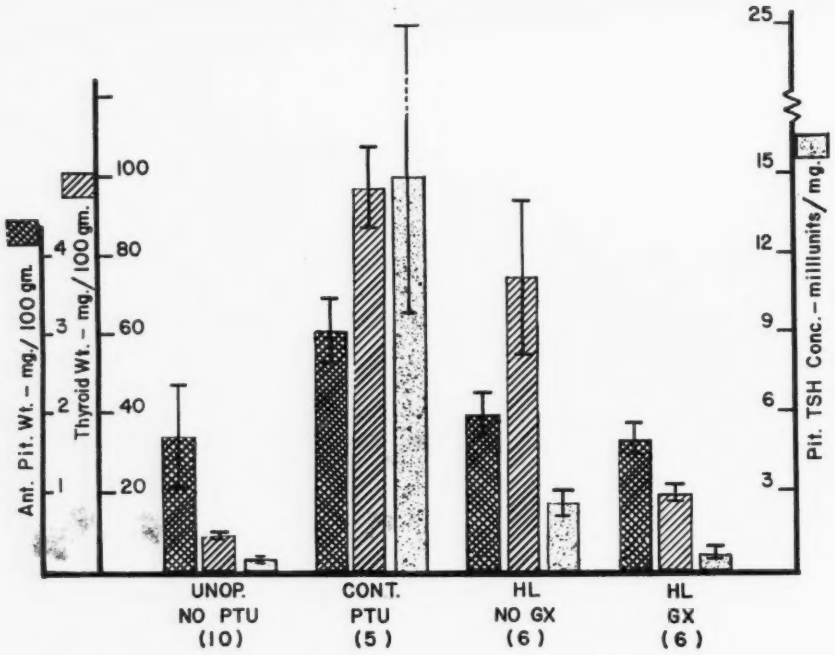

FIG. 5. Chart of mean thyroid and adenohypophyseal weights and adenohypophyseal TSH concentrations for Groups I, IV, VI and V (see Table 1). Vertical lines through tops of bars represent either standard error of mean (for gland weights) or range corresponding to 1 standard error of the mean tadpole thyroid cell height response (for anterior pituitary TSH bioassays).

trophy appreciably (Table 1), but the effects were not statistically significant. Adenohypophyseal weight was also greater in the older PTU controls (Group VII) than in comparable guinea pigs with lesions (Group VIII), but as there were no "euthyroid" controls of similar age and body weight, this difference cannot be evaluated fully.

In Figure 5 the relative thyroid and anterior pituitary weights for Groups I, IV, VI and V of Table 1 are compared with the corresponding adenohypophyseal TSH concentrations. These were 0.45 (0.35–0.55), 15.0 (10–25), 2.5 (1.8–3.0), and 0.6 (.45–.80) milliunits (range) per mg. of wet weight, respectively. The concomitant changes of anterior pituitary size and TSH concentration, and of thyroid size, in guinea pigs receiving chronic PTU-treatment are in accord with previous findings (7, 8). The lesions of Groups V and VI either decreased or abolished these changes. Although the anterior lobe hypertrophy (75%) seen in Group IV was not as great as the increases (>1000%) in thyroid size and anterior pituitary TSH concentration, it should be taken into account in evaluating total TSH stores. These were calculated (mean TSH concentration×mean

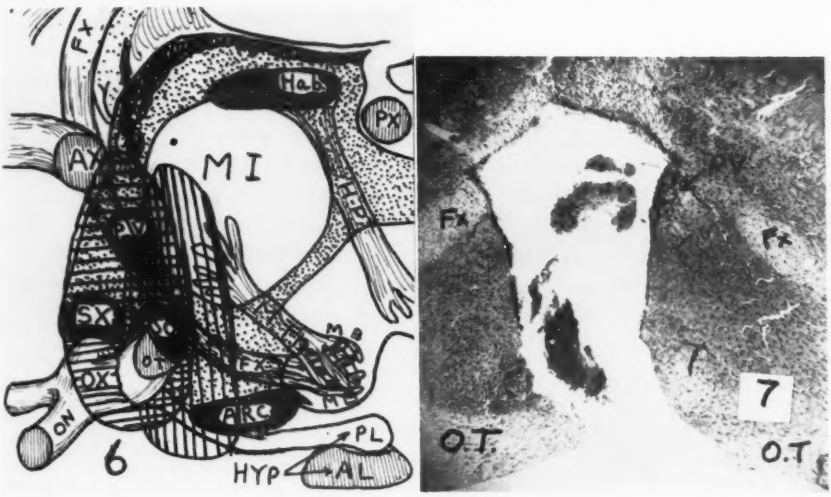

FIG. 6. Diagram of sagittal section of guinea pig diencephalon, with parasagittal structures shown by projection. ON, OX, OT = optic nerve, chiasm and tract; AX and PX = anterior and posterior commissures; MB = mammillary body; MI = massa intermedia; FX = fornix; M-Th = mammillo-thalamic tract; H-P = habenulo-peduncular tract; Hab. = habenular nucleus; P-V = paraventricular nucleus; S-X, S-O = suprachiasmatic and supraoptic nuclei; Arc. = arcuate nucleus; Hyp. = hypophysis (P.L. = posterior lobe, A.L. = anterior lobe); Third ventricle is stippled. The lesions of group V were in the area shown by horizontal hatching. Group VI lesions were in the area shown by vertical hatching.

FIG. 7. Section through a Group V lesion (guinea pig 50, thyroid weight 11.4 mg./100 gm.; testis and seminal vesicle weights were 715 and 185 mg./100 gm., respectively). The lesion did not involve the median eminence. FX = fornix; OT = optic tract.

adenohypophyseal weight) to be about 4.3, 250, 25 and 5.6 U.S.P. milliunits of TSH *per gland* for Groups I, IV, VI and V, respectively.

Gonads. Three lesion-bearing guinea pigs with marked testicular and seminal vesicle atrophy as well as "goiter block" were not included in Table 1. Slight testicular atrophy was seen in 5 of the 6 animals of Group VI.

The gonads of the other animals were well maintained. In fact, seminal vesicle weight was increased significantly ($P < .001$) in Group III, suggesting heightened androgen secretion. This accessory sex gland hypertrophy, cited elsewhere (11), was observed less frequently in the other groups.

Lesion localization. In the 6 animals of Group V, the fairly symmetrical lesions destroyed the region of the paraventricular nuclei and extended between the columns of the fornix from just behind the anterior commissure well into the anterior part of the ventricle (Fig. 7, cf. Fig. 6). They usually reached to the floor of the brain at some point between the optic chiasm and the median eminence.

Similar lesions were found in Group III animals without goiters. Although it cannot safely be said that they caused "goiter block," it is possible to state that no guinea pig with such a lesion exhibited a large goiter.

In general, the "ineffective" lesions were somewhat more posterior (Group VI) (Fig. 6) or else similarly placed but smaller or asymmetrical (Group VIII). Those which caused gonad atrophy were basal and greatly damaged either the infundibulum (2 of the 3 omitted from Table 1) or the arcuate nucleus region. In this connection it is of interest that the testes and seminal vesicles of 5 of the 6 guinea pigs in group VI weighed less than the control (Group IV) average. The lesion leading to gonad atrophy in the male guinea pig thus appears to be similar to that causing gonad atrophy in the male rat (12).

DISCUSSION

These findings clearly indicate that appropriate hypothalamic lesions may produce "goiter block" in the guinea pig, preventing not only most of the thyroid weight increase but also, to a great extent, the microscopic manifestations of goitrogenesis. On the other hand, few lesions prevented an increase of the T/S ratio after PTU treatment and in no instance was this ratio significantly less than the mean ratio for "euthyroid" controls (Fig. 1). Although data are not available on the T/S ratios of PTU-treated, hypophysectomized guinea pigs, it seems reasonable to assume that in such animals the T/S ratio would be less than 57. Taken together with the fact that most of the lesions which affected thyroid structure did not prevent the PTU-induced rise of the T/S ratio (Fig. 2), this seems to indicate that the lesions did not block TSH secretion completely. The T/S ratio in the rat responds to very much smaller amounts of exogenous (13) or endogenous (14) TSH than are needed for thyroid hypertrophy or hyperplasia. If these parameters can be assumed to respond similarly in the guinea pig, it follows that the extent to which TSH release in this species has been impaired by hypothalamic lesions is greater than in the rat (4, 5) but less than in the dog (6).

The "euthyroid" T/S ratio (57 ± 8) found in this study was somewhat higher than that found in rats not treated chronically with goitrogen (4, 9). This is of interest in view of the relatively low level of thyroid activity in the normal guinea pig (8).

The marked reduction of TSH stores in the adenohypophyses of PTU-treated guinea pigs with hypothalamic lesions, whether or not they developed goiters, suggests that TSH synthesis may have been impaired even more than release. This point will require further investigation, however. Since thyroid size is only a crude index of TSH release, there might well have been some degree of "goiter block" even in animals with considerable thyroid enlargement. The determination of circulating TSH levels might be helpful in elucidating this question. In any case it is clear that both the

release and storage of thyrotrophin were decreased markedly by lesions which did not impair gonad maintenance. We interpret this to mean that such lesions can selectively impair TSH *synthesis*, as well as *release*.

This seems to be in contrast with the failure of hypothalamic lesions to prevent TSH reaccumulation by the adenohypophyses of rats from which PTU had been withdrawn (15, 16). Although TSH synthesis in the rat did not appear to be influenced by hypothalamic lesions in this situation, it clearly was impaired under other conditions (5). In the absence of any report on the effects of hypothalamic lesions on the TSH "rebound" phenomenon in the guinea pig, further comparison of the neural influences on TSH synthesis in these two species must be withheld.

It is not yet possible to identify the neural structures which appear necessary for augmented TSH secretion in the guinea pig. As in the rat (1–4, 12) and dog (6) they seem to lie, at least in part, in the anterior hypothalamus. They may be damaged demonstrably by lesions which do not seem to interfere with testicular maintenance and therefore must be somewhat separate from the neural mechanisms concerned with gonadotrophin secretion.

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ANTIGOITROGENIC AND CALORIGENIC ACTIVITIES OF THYROXINE ANALOGUES IN RATS[1]

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ABSTRACT

The relative potencies of more than forty thyroxine analogues and a soluble hog thyroglobulin extract were established in parallel goiter-prevention and calorigenic assays against the same thyroxine standard. By both methods of assay twelve of the preparations had activities ranging from 5% to 800% of that of thyroxine. The activities, if any, of the remaining analogues were less than 3% of that of the standard.

Analysis of area-under-curve and speed of response in calorigenic assays indicated that all the active analogues and soluble thyroglobulin were qualitatively similar with respect to this parameter of thyromimetic function in normal adult male rats. Thyroglobulin was characteristically more active than expected when its potency, relative to thyroxine, was calculated on the assumption that its thyronine-bound iodine, as determined by the Blau, 1935, procedure, was in the form of thyroxine.

Correlations of structural changes with corresponding assay activities indicated the following generalizations: (a) analogues having an intact alanine side chain required iodine substituents at least in the 3 and 5 positions as a condition for biological activity. Thyronines having iodine substituents in the 3, the 3 and 3′, or the 3, 3′ and 5′ positions were biologically inactive at relatively high dose levels; (b) the potency of analogues with deaminated side chains decreased as the chain length increased from two to four carbons; (c) analogues having a deaminated side chain required iodine substituents in the 3 and 5 positions with additional iodine substitution in either or both the 3′ and 5′ positions, as a condition for biological activity, but 3,5,3′-triiodo compounds were not necessarily more active than the corresponding 3,5,3′ ,5′-tetraiodo derivatives; (d) inactive 3,5-diiodothyropropionic acid was activated by introduction of methyl groups in positions 3′ and 5′; (e) replacement of the phenolic hydroxyl group at the 4′ position by the methyoxy group caused a significant reduction in goiter-prevention activity.

Ratios of antigoitrogenic to calorigenic potencies, ranging from approximately 1.0 for 3,5,3′-triiodo-l-thyronine to 5.0 for 3,5,3′,5′-tetraiodothyroacetic acid, were indicative of a real dichotomy of action for some of the analogues.

The decomposition of 3,5,3′,5′-tetraiodothyroacetic acid and two other analogues, in alkaline solution, suggested a note of caution with regard to the interpretation of previously published bioassays for such preparations.

Comparative results showed that in most cases, the calorigenic assay could be used interchangeably with the antigoitrogenic assay to predict the exogenous dose of an analogue equivalent to the daily endogenous hormonal output by normal rat thyroids.

[1] Presented at the 1958 Meeting of the American Goiter Association.

BIOASSAY results, covering forty-five thyroxine analogues, are presented as a contribution toward the increasing effort to delineate the effects of alterations in molecular structure on biological activity (1–5). The difficulties of correlating structure and activity from the reports of many investigators are enhanced by the variety of bioassay methods employed, unreported activity for a given compound by one or more assay methods, and the uncertainty regarding the identity and purity of some of the tested compounds.

This study was designed to overcome the aforementioned difficulties by employing a closely correlated program of organic synthesis and bioassays. Thus, virtually all the analogues were prepared and carefully characterized in these laboratories. Antigoitrogenic and calorigenic assays employing rats of the same strain and source were conducted in these laboratories while Money *et al.* (6, 7) assayed the compounds for I^{131} uptake activities in rats and also for induction of tadpole metamorphosis. Accordingly, comparisons between analogues, assay methods and species could be made with greater confidence.

During the course of this work an unanticipated decomposition of certain thyroxine analogues in alkaline solution was observed. Special attention was therefore given to the critical evaluation of the results of bioassays performed on such compounds.

MATERIALS AND METHODS

Antigoitrogenic Assay

During the fourteen day test period female albino rats, weighing 160–190 gm., were allowed free access to tap water and powdered rat diet containing thiouracil (0.3% by weight). Room temperature was maintained at 75° ± 3° F. Solutions of thyroxine and one or more analogues[2] were assayed simultaneously. Rats were injected subcutaneously, daily, on Days 0 through 13. Five to ten animals were allotted to each dose level and three or more dose levels were employed for each of the active analogues. On Day 14, the thyroid glands were rapidly removed, cleaned, weighed to the nearest 0.1 mg. and recorded for each rat as mg. of gland weight per 100 gm. of body weight. Preparations suspected of having little or no activity were screened at a single dose level which was at least twenty times the dose of thyroxine (4 μg./rat/day) required to produce a 67% inhibition of thiouracil-goiter. Compounds found active by screening were reassayed at three or more dose levels.

Calorigenic Assay

Male albino rats weighing 280–320 gm. were trained to eat their total daily requirement of powdered rat diet between 11:30 A.M. and 4:30 P.M. daily (8) for at least two

[2] Crystalline *l*-thyroxine and 3,5,3′-triiodo-*l*-thyronine were obtained from Cyclo Chemical Corp., Cal. Gifts of the following analogues are gratefully acknowledged: 3,5,3′,5′-tetraiodothyroacrylic acid from Dr. Stanley Wawzonek, State Univ. of Iowa, Iowa City; 3,5-diiodo-3′,5′-dimethylthyro-propionic acid from Dr. Norman Kharasch, Univ. of Southern California, Los Angeles; 3,5,3′,5′-tetrachloro-*dl*-thyronine synthesized by Sir. C. R. Harrington and furnished by Dr. J. Lerman, Boston.

weeks prior to and during the BMR test period. BMR tests consisted of three or six consecutive ten-minute readings of oxygen comsumption for each rat on several days before, during and after drug administration. These determinations were always made between 8:00 A.M. and 11:30 A.M. Thus, the fasting period preceding each BMR test varied between sixteen and nineteen hours. Twelve Benedict-type (9) closed circuits maintained at 29° ±0.2° C made it convenient to run twenty-four to forty-eight rats each morning. The lowest two or four ten-minute readings of the three or six, respectively, which were made for each rat were averaged, corrected to standard conditions and recorded as mg. O_2/Kg. body weight/hour. For the most part, percentage changes in BMR, during and after drug administration, were based on the average pretreatment level for each rat. However, in some assays it was more convenient to use the day to day average of a placebo-injected control group as the basis for calculating the percentage changes of treated groups on the same day. Thyroxine and one or more analogues were assayed concomitantly at two or more dose levels per preparation, using five to twelve rats per dose level. A shortage of one active analogue resulted in tests at a single dose level. The active analogues were injected subcutaneously, once per day, for a period of five to fourteen days. All compounds suspected of having little or no thyroxine-like activity were screened at a single dose level (1 or 2 mg./rat/day/3–4 days, subcutaneously) in groups of four rats per preparation. These dose levels were five to ten times greater than the amount of thyroxine required to produce a strong response.

Solutions

In the early phases of this study preparations were dissolved in a minimum volume of 0.1*N* NaOH and diluted to required concentrations with physiological saline. Subsequently, several analogues were prepared for injection by dissolving them in ethyl alcohol with added 0.1*N* NaOH (if needed). Saline (adjusted to pH 8–9) was used for dilutions, and the final concentration of alcohol was always 10% or less. A solvent consisting of ethyl alcohol/propylene glycol, 10/90, V/V, was required for a compound which was extremely labile in the presence of alkali, under certain conditions. Volumes of solution sufficient to last up to fourteen days were distributed in stoppered vials and maintained frozen between injections. Daily doses for each rat were usually contained in a volume of 0.5 cc. to 1.0 cc. In order to minimize contamination of samples in storage vials, hypodermic needles were never used for drawing doses after the needles had been used for injection.

Computation of Results

In assays designed with two or more dose levels, for standard and unknowns, the regression equation, $Y = a + b \log. X$, was calculated for each preparation (10). The several b-values in a completed experiment were replaced by the average slope (b) with due regard for inequalities in numbers of observations and/or dose increments (11). The potency of each unknown (U) relative to standard (S) was calculated as the antilog. of [av. log. Xs − av. log. Xu + (av. Yu − av. Ys/av. b)]. In addition, the resulting equations were used for the derivation of X-intercept values in each method of assay. Such values for goiter-prevention results were first used by Dempsey and Astwood (12) but their usefulness in the analysis of calorigenic assays appear to have been largely overlooked. When a compound was tested at a single dose level, the amount of thyroxine required to produce the same response was read from the concomitant standard curve. This value, divided by the dose of unknown, gave a relative potency estimate for the preparation. The validity and precision of these assay methods, under the experimental conditions described here, were presented and discussed in a previous report by Stasilli and Kroc (13). Confidence in the results is greatly strengthened by the fact that "standard" was always tested concomitantly with each unknown in homogeneous groups of rats. Thus, variations in results which might be ascribed to species, sex, weight or

seasonal and environmental differences, etc., (2) were either nullified or greatly diminished.

RESULTS AND DISCUSSION

Active analogues—relation of structure and activity:

Typical calorigenic response curves for thyroxine, some of its analogues and soluble thyroglobulin are shown in Figures 1 and 2. It is apparent that

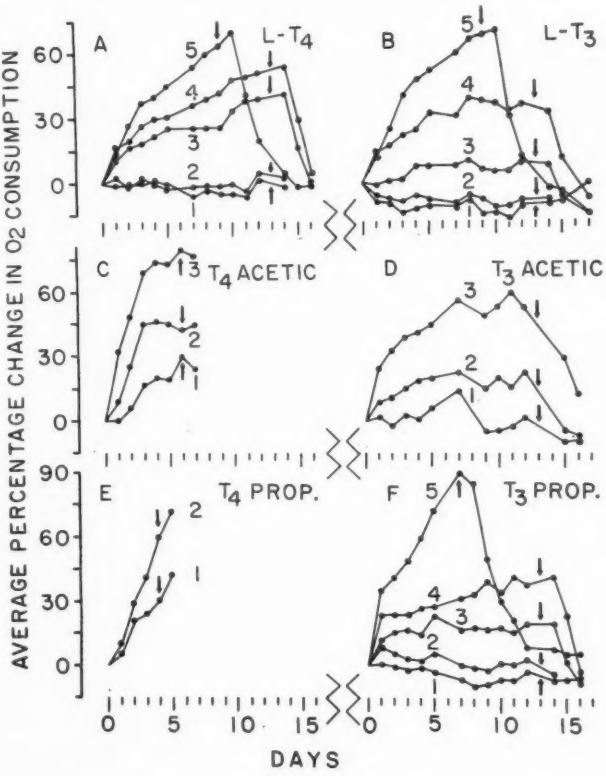


FIG. 1. *BMR response of normal adult male rats during, and after, 5–14 days subcutaneous injections of thyroxine analogues (μg./100 gm. body weight/day):*

A. L-Thyroxine (sodium pentahydrate): 1, alkaline-saline controls; 2, 2 μg.; 3, 6 μg.; 4, 16 μg.; 5, 55 μg.

B. L-Triiodothyronine: 1, alkaline-saline controls; 2, 0.3 μg.; 3, 1 μg.; 4, 3 μg.; 5, 10 μg.

C. 3,5,3′5′-tetraiodothyroacetic acid (in alcohol-propylene glycol): 1, 50 μg.; 2, 150 μg.; 3, 450 μg.

D. 3,5,3′-triiodothyroacetic acid: 1, alkaline-saline controls; 2, 30 μg.; 3, 105 μg.

E. 3,5,3′,5′-tetraiodothyropropionic acid: 1, 420 μg.; 2, 1270 μg.

F. 3,5,3′-triiodothyropropionic acid: 1, alkaline-saline controls; 2, 30 μg.; 3, 90 μg.; 4, 300 μg.; 5, 900 μg.

Last daily injection shown by arrow.

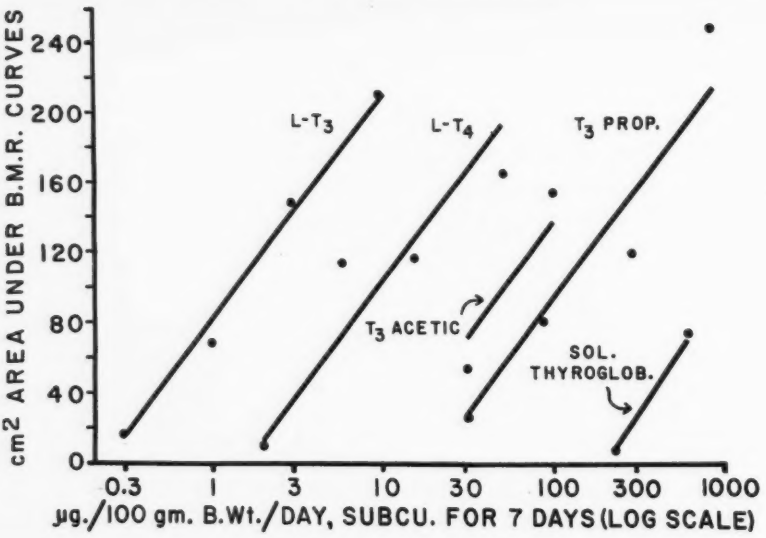
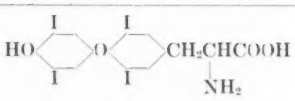
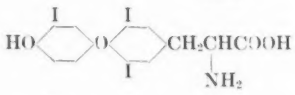
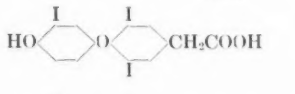
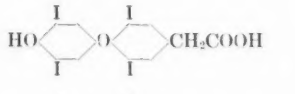
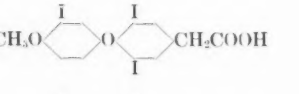
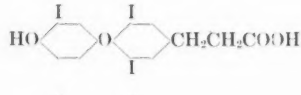
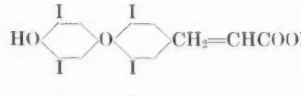
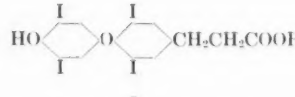
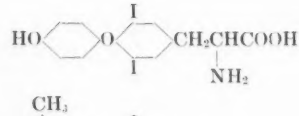
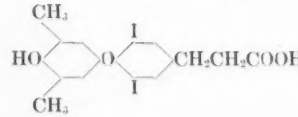
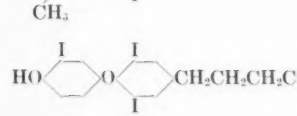
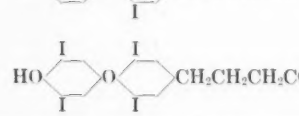


FIG. 2. *Analysis of areas under BMR curves for thyroxine analogues and soluble hog thyroglobulin:* Each curve in Figure 1 was plotted on linear graph paper such that 1 inch on the ordinate scale equaled 10 percentage points of BMR increase and 1 inch on the abscissa was equal to 1 day. The total area under each curve was determined with a polar planmeter from Day 0 through the Day 8, where applicable. (Similar area analyses for Figure 1C and 1E were made but not shown.) Two or more points, obtained in this manner for each preparation, were plotted against the respective dose levels. Calculations for the best fitting straight lines (Y = area versus X = log. dose) were made as explained in text. A common slope value was used for each line.

the various preparations affected the oxygen consumption of rats in much the same manner. The major differences seen were among the doses of individual drugs required to produce a given level of response. These differences were more accurately assessed by determining the area-under-curve at all dose levels of each compound for a given number of treatment days. The areas and the logarithms of their corresponding doses were used to compute the best fitting straight lines (with common slope) shown in Fig. 2.

The potency of each unknown, relative to thyroxine, is listed in Table 1 where the results by goiter-prevention test are also entered for comparison of the assay methods. It will be noted that the potency values are presented in the order of decreasing activity by goiter-prevention test for all active analogues other than thyroxine. It is apparent that the calorigenic activity values are in general agreement with the goiter-prevention activity values, as to order. The activity of 3,5,3′-triiodo-*l*-thyronine was, as expected, greater than thyroxine and all the analogues. The tri- and tetraiodothyroacetic acids were antigoitrogenically equal to each other and

TABLE 1. ACTIVE THYROXINE ANALOGUES

			Relative activities	
			Goiter Prevention	Calorigenic
W1563		L-thyroxine (sodium pentahydrate)	100	100
W1575		3,5,3′-triiodo-l-thyronine	800	800
W1499		3,5,3′-triiodothyroacetic acid	51	21
W1483		3,5,3′,5′-tetraiodothyroacetic acid	57	11
W1702		3,5,3′-triiodothyroacetic acid, methyl ether	32	11
W1525		3,5,3′-triiodothyropropionic acid	28	10
W1745		3,5,3′,5′-tetraiodothyroacrylic acid	20	13
W1524		3,5,3′,5′-tetraiodothyropropionic acid	14	7
No. 37		3,5-diiodo-l-thyronine	>7	11*
W1659		3,5-diiodo-3′,5′,-dimethylthyropropionic acid	8	5
W1621		3,5,3′-triiodothyrobutyric acid	7	5
W1620		3,5,3′,5′-tetraiodothyrobutyric acid	7	5

* Value reported based on results with single dose level.

about half as active as thyroxine. Pitt-Rivers (14) also found these two analogues equally potent by goiter-prevention assay. As may be seen, at the bottom of Table 1 the tri- and tetraiodothyrobutyric acids were also equally potent. These results suggest that introduction of the fourth iodine at the 5′ position does not necessarily result in a reduction of activity in homologous series with deaminated side chains. Calorigenically, 3,5,3′-triiodothyroacetic acid appears to be more potent than its tetraiodo homologue. The difference is of borderline significance statistically. However, Barker (15) observed a similar difference in calorigenic activity for the tri- and tetraiodothyroacetic acids. The importance of the phenolic hydroxyl group in the 4′ position is reflected in the significant reduction of goiter-prevention activity of compound W1702 compared to compound W1499. The drop in activity resulted from replacement of the 4′ hydroxyl group, in 3,5,3′-triiodothyroacetic acid, with a methoxy group. The effects of side chain length on activity are also shown in Table 1. Activity decreased uniformally as the deaminated side chain length was increased from two to four carbons. Thus, the "propionic," "acrylic" and "butyric" acid analogues were each less active than the "acetic" acid analogues. This finding appears to conflict with a postulate of Selenkow *et al.* (3) which suggests decreasing activity with decreasing side chain length. It is true, however, that the "benzoic acid" analogues do not possess appreciable activity by these assay methods (Table 2) (16, 17).

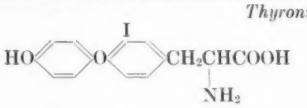
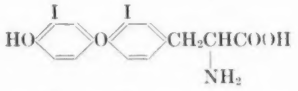
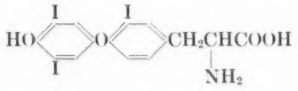
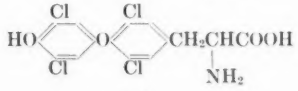
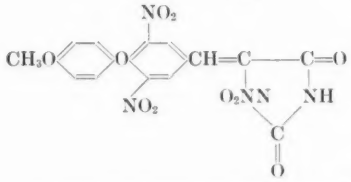
The importance of iodine and its structural position with relation to activity can be seen by the assay results listed in Tables 1 and 2. It will be seen that in the thyronine series, 3-monoiodo and 3,3′-diiodo-*dl*-thyronine were inactive (Table 2) but 3-5-diiodo-*l*-thyronine had significant activity (Table 1). This latter result agrees with Selenkow *et al.* (3). In the "acetic acid" and "propionic acid" series the 3-monoiodo, 3,3′-diiodo and 3,5-diiodo preparations were inactive (Table 2). Similarly, 3,5-diiodothyrobutyric acid was found to be inactive. Therefore, it appears that, in the thyronine series, iodines in the 3,5 positions are required for significant biological activity. For preparations with deaminated side chains, iodines in positions 3,5,3′ or 3,5,3′,5′ are needed for biological activity. However, an exception to this latter observation may be seen in Table 1 where 3,5-diiodo-3′,5′-dimethyl-thyropropionic acid is shown to have significant activity. Thus, the inactive 3-5-diiodothyropropionic acid (Table 2) can be rendered active by introduction of methyl groups at the 3′ and 5′ positions (W1659, Table 1).

Active analogues—relation of antigoitrogenic to calorigenic activities

An interesting feature of Table 1 is the greater goiter-prevention potencies of several analogues compared to their respective calorigenic activities. Ratios of goiter-prevention/calorigenic assay values of these analogues are: tetraiodothyroacrylic, 1.5; tetraiodothyropropionic, 2.0; triiodothyro-

TABLE 2. ANALOGUES ASSAYING LESS THAN 3% OF LEVO THYROXINE BY GOITER-PREVENTION AND CALORIGENIC TESTS

Thyronine series

W1631		3-iodo-dl thyronine
W1641		3,3′-diiodo-dl-thyronine
W1646		3,3′,5′-triiodo-dl-thyronine
W1633		3,5,3′,5′-tetrachloro-dl-thyronine
No. 12		1-nitro-5-(3,5-dinitrothyromethylene) hydantoin, methyl ether

Benzoic acid series

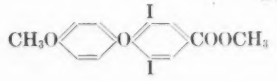
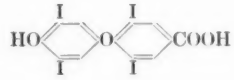
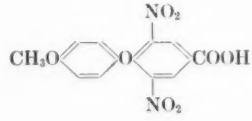
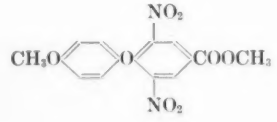
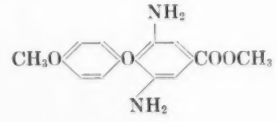
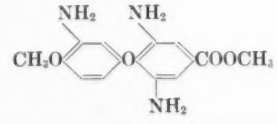
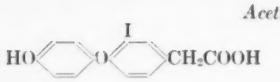
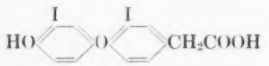
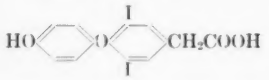
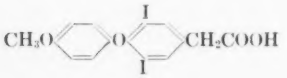
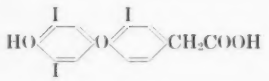
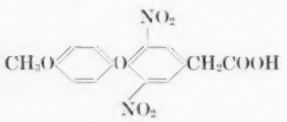
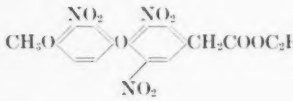
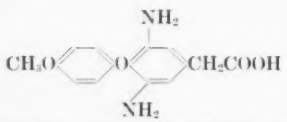
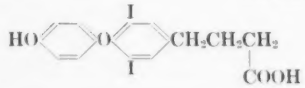
No. 6		3,5-diiodothyroformic acid, methyl ether, methyl ester
W1489		3,5,3′,5′-tetraiodothyroformic acid
No. 4		3,5-dinitrothyroformic acid, methyl ether
No. 5		3,5-dinitrothyroformic acid, methyl ether, methyl ester
No. 9		3,5-diaminothyroformic acid, methyl ether, methyl ester
No. 11		3,5,3′-triaminothyroformic acid, methyl ether, methyl ester

TABLE 2. *(Continued)*

Acetic acid series

W1691		3-iodothyroacetic acid
W1701		3,3′-diiodothyroacetic acid
W1502		3,5-diiodothyroacetic acid
W1680		3,5-diiodothyroacetic acid, methyl ether
W1699		3,3′,5′-triiodothyroacetic acid
No. 10		3,5-dinitrothyroacetic acid, methyl ether
No. 2		3,5,3′-trinitrothyroacetic acid, methyl ether, ethyl ester
No. 15		3,5-diaminothyroacetic acid, methyl ether

Butyric acid series

W1618		3,5-diiodothyrobutyric acid

Propionic acid series

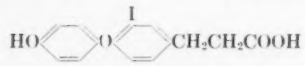
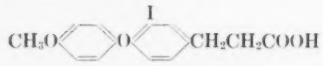
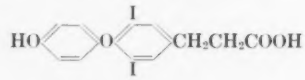
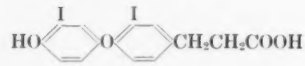
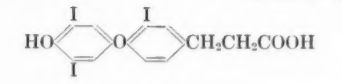
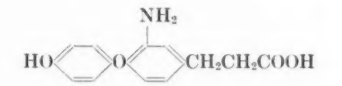
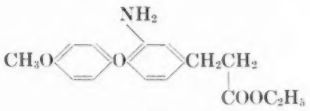
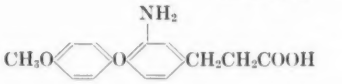
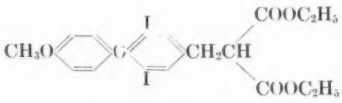
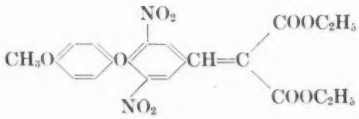
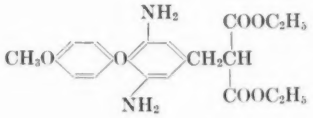
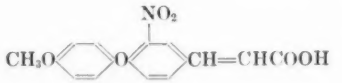
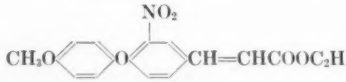
W1608		3-iodothyropropionic acid
No. 22		3-iodothyropropionic acid, methyl ether
W1607		3,5-diiodothyropropionic acid
W1622		3,3′-diiodothyropropionic acid

TABLE 2. (*Continued*)

W1628		3,3′,5′-triiodothyropropionic acid
No. 20		3-aminothyropropionic acid
W1704		3-aminothyropropionic acid, methyl ether, ethyl ester
No. 51		3-aminothyropropionic acid, methyl ether
No. 17		3,5-diiodothyromethylmalonic acid, methyl ether, diethyl ester
No. 8		3,5-dinitrothyromethylenemalonic acid, methyl ether, diethyl ester
No. 16		3,5-diaminothyromethylmalonic acid, methyl ether, diethyl ether
No. 21		3-nitrothyroacrylic acid, methyl ether
No. 24		3-nitrothyroacrylic acid, methyl ether, ethyl ester

acetic, 2.4; triiodothyropropionic, 2.8; methyl ether of triiodothyroacetic, 2.9; and tetraiodothyroacetic, 5.0. With due regard for the error of estimate in each assay method, it can be stated that ratios of 2.5 or greater may be considered statistically significant at $p = 0.95$. It is interesting that these same preparations were the ones found to be most active by the tadpole metamorphosis assay, by Money *et al.* (6, 7). Such high ratios may represent a real dichotomy of actions, for example with respect to suppression of pituitary TSH output versus stimulation of oxygen consumption, within a given species (rats), as well as between species (rats versus tadpoles).

Another possible cause of high ratios may relate to the differential calorigenic activities of several analogues in normal versus thiouracilized rats. This latter point will be the subject of a subsequent paper (18).

Active analogues—decomposition associated with method of solution

The results of repeated assays with 3,5,3′,5′-tetraiodothyroacetic acid are shown in Table 3. This close examination was made to verify the previously mentioned high goiter-prevention/calorigenic activity ratio of 5.0 and also to account for a peculiar behavior of this analogue under certain conditions of solution. In the first four assays (Table 3) the compound was dissolved in alkali and diluted with physiological saline. The resulting potencies of 56% and 69% by goiter-prevention test were observed with groups of five to eight rats each, at several dose levels. Calorigenically, the same composite lot assayed 6% in one group of four rats at a single dose level, and 11% with groups of eight rats at three dose levels. The high ratio between activities by the different assay methods was thus firmly established. After these results were obtained the method of solution was modified by using alcohol as a primary solvent, followed by alklali and physiological saline as a diluent. Many of the inactive analogues (Table 2) under test at that time were more easily solubilized by this procedure. Solutions of a new lot of tetraiodothyroacetic acid (lot 6, assays 56 and 56A, Table 3) were prepared for tests using these modifications. The compound was par-

TABLE 3. EFFECTS OF METHOD OF SOLUTION ON BIOLOGIC ACTIVITY OF 3,5,3′,5′-TETRAIODOTHYROACETIC ACID. (W1483, T_4 ACETIC)

Assay No.	Lot No.	Per cent of thyrox. activity		Method of solution
		Goiter-prevention	Calorigenic	
36A	Composite (2, 3, 4)	56	—	Alkaline-saline[1]
46A	Composite (2, 3, 4)	—	6*	Alkaline-saline[1]
43	Composite (2, 3, 4)	69	—	Alkaline-saline[1]
49A	Composite (2, 3, 4)	—	11	Alkaline-saline[1]
56; 56A	6	—	<1†	Alcohol-alkaline-sal.[2]
51	2	50	—	Alcohol-alkaline-sal.[2]
51	3	54	—	Alcohol-alkaline-sal.[2]
51	4	52	—	Alcohol-alkaline-sal.[2]
51	6	47	—	Alcohol-alkaline-sal.[2]
62A	6	—	11	Alcohol-prop. glycol.[3]
53	6	58	—	Alcohol-prop. glycol.[3]
53	6	69	—	Alcohol-alkaline-sal.
53	6	<1†	—	Alcohol-alkaline-sal.
Average (omitting* and †):		57	11	
Range of mean (P=0.95):		50–64		

* Preliminary test: four rats: single dosage level.

† Solutions developed color reaction.

[1] Preps. dissolved in minimal alkali (N/10 NaOH) and diluted with physiological saline.

[2] Preps. dissolved in absolute ethyl alcohol (plus minimal volume of N/10 NaOH, if needed) and diluted with saline adjusted to pH 8–9. Alcohol not greater than 10%, by volume, in final solution.

[3] Preps. dissolved in absolute ethyl alcohol and diluted with propylene glycol, such that final solution was 10% and 90%, respectively, by volume.

tially dissolved by alcohol. The addition of a small volume of N/10 NaOH gave a clear amber-colored solution. When saline adjusted to pH 8–9 was added as diluent, the color of the resulting solution immediately changed to dark green. Within two minutes the color gradually returned to a clear yellow. During the course of five days of administration of this solution in a group of eight rats, no significant elevation of BMR was obtained although, in the concentration employed, the drug was expected to produce a strong response. Thus, the activity, if any, was recorded as less than 1%, as shown in Table 3.

The possibility that lot 6 might be chemically different from the composite of lots 2, 3 and 4 (Table 3, first four assays) was considered in spite of the fact that paper partition chromatography indicated that the various lots were quite similar. A new solution of lot 6, in a concentration three times greater than previously employed, was prepared using the alcohol-alkali-saline system as before. The same color changes were observed, and assay confirmed the essential lack of calorigenic activity (Table 3, No. 56A). Lots 2, 3, 4 and 6 were then assayed separately for goiter-prevention activity. Solutions, at the much lower concentrations needed in this test method, were prepared with the alcohol-alkali-saline system. No color changes were observed and, as shown by assay No. 51, Table 3, the four lots had the expected activities. The differences in activities between lots were therefore not significant, thus corroborating observations made by means of chromatographic analysis.

At this point, it was apparent that we were dealing with a preparation for which a valid assay of biological activity depended on a set of specific conditions including the drug/alkali ratio. In order to test this hypothesis, lot 6 was again assayed, by both methods, under three different conditions of solution. For calorigenic assay No. 62A, Table 3, 675.0 mg. of lot 6 were dissolved in 15 cc. absolute ethyl alcohol and diluted to 150 cc. with propylene glycol. Since no alkali was present no color change was observed. Doses of 50 μg., 150 μg. and 450 μg./0.25 cc./100 gm. body weight were administered subcutaneously daily, for seven days, using eight rats per dose level. When the areas under the resulting BMR increment curves were used to construct a dose-response curve and this was compared to the curve for the thyroxine standard, the calculated relative activity of lot 6 was 11%. This confirmed earlier assays (Nos. 46A and 49A, Table 3) of alkaline solutions of identical drug concentrations, which did not give color reactions. For goiter-prevention assay No. 53, Table 3, lot 6 was prepared in three ways. Twelve milligrams were dissolved in 50 cc. of alcohol and a 25 cc. aliquot thereof was diluted to 250 cc. with propylene glycol to complete the first solution. A second solution was prepared by addition of 2.5 cc. N/10 NaOH to the remaining 25 cc. of the alcoholic solution followed by dilution to 250 cc. with saline which had been previously adjusted to pH 8–9. No color change was anticipated and none was observed.

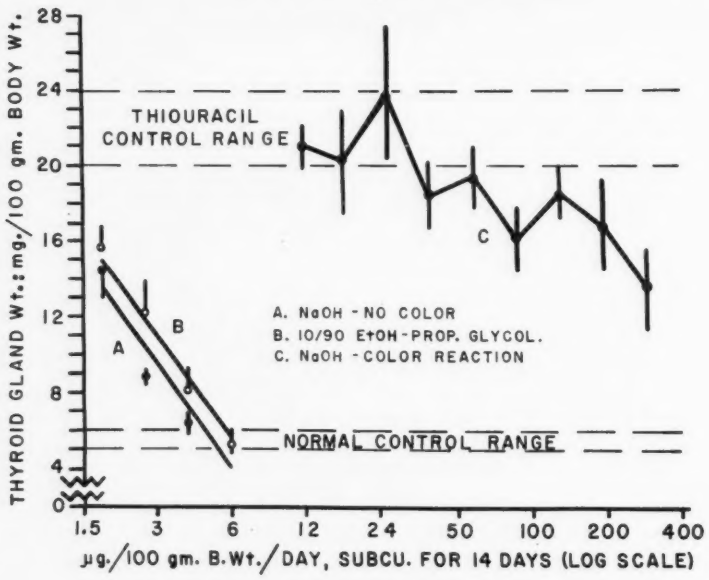


FIG. 3. *Effect of method of solution on goiter-prevention activity of 3,5,3',5'-tetraiodothyroacetic acid:*

A. Alkaline-saline solution, pH above 11 (no color reaction).

B. Alcohol-propylene glycol solution (no alkali, no color reaction).

C. Alkaline-saline solution, pH substantially below 11 (color reaction developed).

The same lot, No. 6, was used to prepare each solution. See text for explanation of concentrations and color reactions.

The third solution, in which a color reaction was deliberately allowed to develop, was prepared by dissolving 180 mg. of lot 6 in 4.5 cc. alcohol and 3.6 cc. N/10 NaOH. Upon addition of 18 cc. of saline (pH 8–9) the expected color changes from amber to dark green and back to yellow were observed. No further change was obtained with addition of more alkali (3.3 cc.) and saline (15.6 cc.). Each of the three solutions was then assayed for goiter-prevention activity, at four or more dose levels, in groups of five to eight rats per dose level. The observed relative potencies are shown in Table 3, assay No. 53 and the dose-response curves were plotted in Figure 3. Curves A and B in Figure 3, representing solutions with and without alkali, respectively (each without color reaction) were not significantly different statistically. The relative potency estimates were 69% for the alkaline solution and 58% for the alcohol-propylene glycol solution as shown in Table 3. These results were in excellent agreement with all previous goiter-prevention assays of the various lots of tetraiodothyroacetic acid. On the other hand, Curve C, Figure 3, clearly demonstrates the expected loss of activity in a solution which gave the color changes. Bioassay calcu-

lations showed that more than 99% of the activity of this latter solution had been destroyed. This finding was substantiated by the fact that none of the original tetraiodothyroacetic acid in this solution could be detected by paper partition chromatography. Whether the minute residual activity (<1%) was due to traces of intact tetraiodothyroacetic acid, which might escape chromatographic analysis, or to biologically active decomposition products remains to be determined.

A more detailed study of the decomposition of tetraiodothyroacetic acid revealed that the speed of degradation depended on a number of variables. Among these were drug concentration, temperature, pH and trace impurities in the various lots of material (Table 3). Decomposition was effectively inhibited by the addition of hydroquinone or thyroxine and/or by lowering the temperature. It was not inhibited when an atmosphere of nitrogen and a solvent free of dissolved oxygen were used. Solutions made up at pH 11–12 and quickly frozen did not show significant decomposition. However, when the initial pH was approximately 9–10, a rapid degradation ensued, at room temperature, and the characteristic color change was obtained. Variations in pH produced concomitant variations in the speed of decomposition. Thus it was possible to obtain slow but significant destruction *without* noticeable color changes. Paper partition chromatographic analysis of such solutions revealed that the original drug had been replaced by several decomposition products including ionic iodide. Observations with other analogues indicated that N-acetylthyroxine and 3,5,3′,5′-tetraiodothyropropionic acid were similarly labile. On the other hand, triiodothyronine, thyroxine, triiodothyroacetic acid and triiodothyropropionic acid were stable under these conditions. A search of the older literature revealed that in the course of work on the structure of thyroxine, Kendall and Osterberg (19) also noted evidence of decomposition. They found that N-acetylthyroxine, unlike thyroxine itself, was unstable as its sodium salt either in solution or in the solid state.

A review of the materials and methods section of many published reports revealed that bioassays of parenterally-administered thyroxine analogues, in animals and humans, have usually been conducted with the use of alkaline solutions (2, 3 and this report). In at least one instance (5) 3,5,3′,5′-tetraiodothyroacetic acid, in alkaline solution, was autoclaved prior to injection. In view of the labile nature of certain analogues, under these conditions, it is suggested that the reported biological actions, or lack of action, attributed to these analogues may be in error. Paper partition chromatographic analysis of test solutions at the beginning and end of injection periods would appear to be a valuable adjunct to the bioassay procedures. For preparations such as 3,5,3′,5′-tetraiodothyroacetic acid, the use of solutions containing no alkali would increase confidence in the results of bioassays.

Evaluation of the decomposition problem

Evidence for the decomposition of one lot of tetraiodothyroacetic acid, in the presence of alkali, appeared late in the sequence of experiments reported here. Since most of the compounds had been dissolved in this manner, it became necessary to determine whether the other analogues, active as well as inactive, had undergone degradation. Accordingly, samples of all of the iodine-containing compounds were again dissolved in alcohol-alkaline saline and studied chromatographically[3] before and after four or five days storage in the frozen state. The pH of the freshly prepared solutions ranged from 7.8–9.8.

As expected, tetraiodothyroacetic acid (W1483, lot 6) and tetraiodothyropropionic acid (W1524) showed marked decomposition. In addition, tetraiodothyroacrylic acid (W1745), tetraiodothyrobutyric acid (W1620) and 3,5,3′-triiodothyrobutyric acid (W1621), each biologically active as shown in Table 1, may have undergone some changes. Similarly, the biologically inactive 3,5-diiodothyroacetic acid, methyl ether (W1680, Table 2) showed some evidence of decomposition. The remaining 22 analogues appeared to be stable in this chromatographic study both initially and after storage.

Since no decomposition had been observed in alcohol/propylene glycol solution, it was of interest to determine the activities of labile compounds in the absence of alkali. Comparative assays of W1483 (Table 3, Fig. 3) indicated that this compound was not more active in alcohol/propylene glycol than it was in alkaline solution when decomposition was effectively inhibited with excess alkali. Similarly, inactive W1680 (Table 2) was again found inactive when dissolved in alcohol/propylene glycol. Higher relative potencies for W1524, W1745, W1620 and W1621 (Table 1) might result if these analogues were assayed in the absence of alkali. If the degree of decomposition for a given compound is not greater than 15%, the observed potencies (Table 1), would not change appreciably because the inherent error of the goiter-prevention assay is $\pm 15\%$ at the 95% confidence limits.

The *in vitro* studies of Freinkel, Ingbar and Dowling (37) indicated that adsorption of thyroxine on glass surfaces might have influenced the outcome of their work with thyroxine-binding protein and thyroxine uptake by tissue. They used physiological levels of thyroxine (about 0.05 μg. to 0.1 μg./ml.) so that even slight adsorption might represent a significant proportion of the total thyroxine present. Previously, the same authors (38) had calculated that 40% of a 200 μg.% saline solution of thyroxine was adsorbed to glassware and that the percentage adsorbed decreased with increasing concentrations.

[3] Circular paper partition chromatography, using Whatman No. 1 paper, was carried out on the samples. The solvent employed was t-amyl alcohol saturated with 6N ammonia.

Adsorption varies with different compounds. The lowest concentrations of compounds used in our experiments were generally greater than 200 μg.%. Bearing in mind the ±15% accuracy of the goiter-prevention test, adsorption of some of the compounds may have occurred, particularly at lower concentrations. Lack of significant adsorption may be postulated, since solutions of the same compound (thyroxine, Table 4) at different concentrations gave essentially the same results. However, preferred procedure would attempt to control the uncertainties and variables of adsorption by equilibrating glassware with preliminary solutions of all compounds.

Active analogues—speed of calorigenesis

The speed of reaction of thyroxine analogues on oxygen consumption, *in vivo* and *in vitro*, has been the subject of conflicting reports. In humans,

TABLE 4. COMPARISON OF "ENDPOINT" DOSAGE IN TWO BIOASSAY PROCEDURES

		Goiter-prevention[1]		Calorigenic[1]	
		No. of assays	Dose[2]	No. of assays	Dose[3]
W1563	L-thyroxine sodium pentahydrate	12	3.3	3	2.3
W1575	3,5,3′-triiodo-l-thyronine	2	0.5	2	0.25
W1499	3,5,3′-triiodothyroacetic acid	2	7.5	2	10.7
W1483	3,5,3′,5′-tetraiodothyroacetic acid	8	5.6	3	21.4
W1525	3,5,3′-triiodothyropropionic acid	3	7.7	1	19.0
W1524	3,5,3′,5′-tetraiodothyropropionic acid	1	22.5	1	37.5

[1] Dose-response curves calculated with 5–12 rats/dosage level and minimum of two dosage levels per prep.
[2] X-intercept: minimum μg. prep./100 gm. body wt./day required to maintain "normal" (6.0 mg./100 gm. B. wt.) thyroid gland weight in 200 gm. Female rats maintained on diet containing 0.3% thiouracil.
[3] X-intercept: maximum μg. prep./100 gm. B. wt./day which will not increase the oxygen consumption of 300 gm. normal male rats.

Blackburn *et al.* (20) found that *l*-triiodothyronine produced a peak response of BMR in 24 to 48 hours versus 7 to 10 days for *l*-thyroxine, while Rall *et al.* (21) observed no qualitative differences in metabolic changes induced by thyroxine, triiodothyronine and tri- and tetraiodothyroacetic acid. The latter two analogues were reported to cause an immediate rise in oxygen consumption, *in vitro*, by Thibault and Pitt-Rivers (22). However, Barker and Lewis (23) observed no immediate metabolic increments for these two preparations either *in vitro* or *in vivo*. Parts of the curves in Figure 1 were used to construct Figure 4 in order to show, more clearly, the comparative BMR responses obtained twenty-four hours after the initial dose of several analogues and a soluble hog thyroglobulin preparation. It is apparent that the responses were roughly proportional to the dose and that each material was capable of producing significant BMR elevation as early as twenty-four hours after injection (Fig. 4, solid bars). Returning to Figure 1, it will be noted that after the final injection (indicated by arrow)

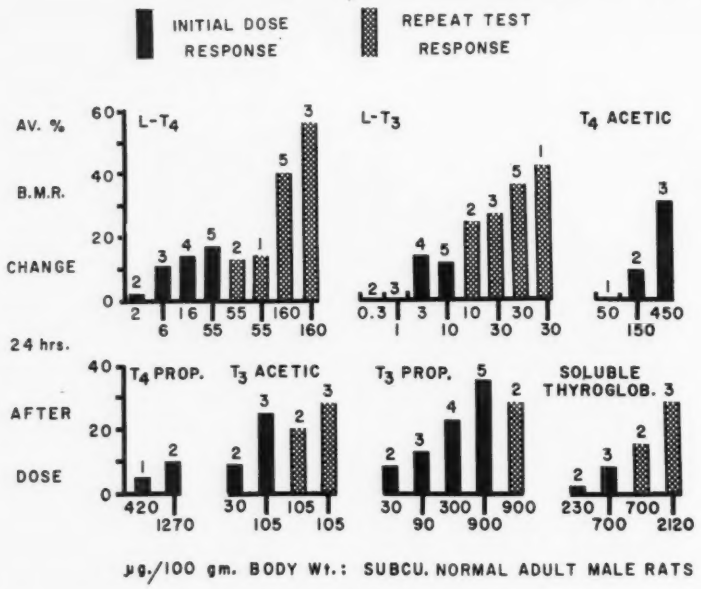


FIG. 4. *Rise in BMR, from normal base line, 24 hrs. after injection of thyroxine analogues and soluble thyroglobulin:*

Solid bars indicate responses on Day 1 shown in Figures 1 and 2.

Symbols at upper left of each series of bars identify compounds for reference to Figures 1 and 2.

Numbers over bars identify specific curves in Figure 1.

Numbers under bars identify the doses.

Cross hatched bars show the 24 hr. responses obtained in repeat tests after various groups in Figure 1 were allowed to return to their normal levels.

the rate of return to pre-treatment base lines was rapid and similar for all preparations. When the rats returned to or near their normal base line they were re-injected with doses equal to or greater than those employed originally and the twenty-four hour response was observed again as shown by the cross-hatched bars in Figure 4. These results indicate that comparable doses produced responses equivalent to those observed initially and that increased dosage resulted in greater responses for each preparation. Therefore, on the basis of BMR increments, determined twenty-four hours after dosing, no qualitative differences in response were observed with any of the materials in rats. However, it is possible that observations made earlier than twenty-four hours (e.g., 6 to 12 hours after injection) might uncover such differences since significant BMR increases have been noted in intact rats (24) as early as twelve hours after injection of soluble thyroglobulin.

The potency of soluble thyroglobulin (Figs. 2 and 4) was only 0.8% of

that of thyroxine when the doses shown were used for calculation. However, this natural hormone contained less than 1% total thyroidal iodine and the biologically active thyronine-bound iodine, as determined by the method of Blau (25), represented less than one-third of the total iodine. This observation, previously reported by Kroc *et al.* (26), supported earlier results obtained by Roche and Michel (27) with thyroid glands of several mammalian species. The thyroxine standard used in this study contained about 55% total iodine and virtually all of this was biologically active. Thus, when the potency was calculated on the basis of thyronine-bound iodine contents, the activity of soluble hog thyroglobulin was 225% of that of thyroxine. Calculations indicate that a minimum of 20 to 25% of the thyronine-bound iodine in the thyroglobulin would have to be present in the form of 3,5,3′-triiodothyronine in order to account for the significant excess of biological activity.

Active analogues—antigoitrogenic and calorigenic maintenance levels

Several years ago, Dempsey and Astwood (12) showed that the maintenance level of thyroxine in rats (or the amount of thyroxine equivalent to the endogenous daily production of normal thyroids), under various environmental conditions, could be accurately assessed by the goiter-prevention method of assay. These results were obtained by noting the point at which a particular dose-response curve intersected a horizontal projection at the level of normal control thyroid weight on the ordinate. Alternatively, the point could be calculated precisely by substituting the normal control weight for Y and solving for X in the regression equation for the dose-response curve. The results thus obtained for thyroxine have been confirmed many times during the ensuing years and the procedure has achieved universal acceptance as a way of determining the activities of thyroidally active materials. More recently the precision of a relative potency estimate by this assay method was observed to be of the order of ±15% at the 95% confidence level (13).

The technical difficulties involved in BMR measurements in rats has generally precluded the usefulness of this assay method for determining maintenance or threshold levels of thyroidal preparations. Such difficulties may account for the wide difference (about 100-fold) in the thresholds of thyroxine-response for the pituitary-thyroid axis versus oxygen consumption, observed by Kennedy and Griesbach (28). Under optimal test conditions the calorigenic assay can yield values, in terms of absolute units, which agree with similar values derived antigoitrogenically. This has been demonstrated by Dempsey and Astwood (12) and Reineke *et al.* (29). Several of the goiter-prevention and calorigenic X-intercept values (bioassay "endpoints") obtained in our studies are shown in Table 4. It will be seen that results obtained by the two assay methods are in good agreement for compounds W1563, W1575, W1499 and W1524 when due

allowance is made for a ±15% goiter-prevention assay error and a −33% to +50% error for the calorigenic assay. It is interesting that the latter assay gave lower results for thyroxine and triiodothyronine. The differences observed for preparations W1483 and W1525 (Table 4) were significant and appear to represent a real dichotomy of action with respect to the two parameters of function (pituitary-thyroid axis versus total metabolism). These results agree with those described in a previous section of this report.

Inactive analogues—antigoitrogenic and calorigenic tests

Thirty-three of the forty-five thyroxine analogues covered by this report were observed to have less than 3% of thyroxine-like activity, if any, as shown in Table 2. On scanning this list it will be noted that all 3- monoiodo and 3,5-diiodo compounds, which have no substituents in the 3′ and/or 5′ positions, were inactive. 3,5-diiodo-*l*-thyronine (Table 1) was the only exception to this rule. Substitution with nitro or amino groups at positions 3; 3,5; or 3,5,3′ gave inactive compounds. Similarly, 3,5,3′,5′-tetrachloro-*dl*-thyronine was found inactive. Several analogues (Table 2) having a 4′-methoxy group instead of a 4′-hydroxyl group were also tested and all were without detectable activity. This was anticipated in view of the significant reduction in goiter-prevention activity obtained with the 4′-methyl ether corresponding to 3,5,3′-triiodothyroacetic acid (Table 1).

Of particular interest in Table 2 was the fact that all 3,3′-diiodo and 3,3′,5′-triiodo analogues were singularly devoid of biological activity by either test. This observation was of special importance with regard to preparations W1641 (3,3′-diiodo-*dl*-thyronine) and W1646 (3,3′,5′-triiodo-*dl*-thyronine) since Roche, *et al.* (30) found the levo-isomers of these compounds to be 85% and 10%, respectively, as active as *l*-thyroxine by goiter-prevention test. In another paper Roche and Michel (31) reported 85% and 5%, respectively, for the *dl*-isomers of 3,3′-diiodo and 3,3′,5′-triiodothyronine versus *dl*-thyroxine by the same method of assay. Pitt-Rivers (32) found no antigoitrogenic activity with a sample of our 3,3′-diiodo-*dl*-thyronine. Similarly, Gemmill (33) observed no oral activity, calorigenically, with his own 3,3′-preparation. Recently, however, Michel (34) found that a sample of our 3,3′-preparation was of equal activity to his own 3,3′-compound by tadpole metamorphosis test, and in his goiter-prevention assay he found 30% to 40% activity, versus *dl*-thyroxine, for our material.

Michel (34) pointed out the possibility that findings of "no" biological activity for 3,3′-diiodothyronine could be due to the unusually rapid action, degradation and excretion of this analogue. Indeed, Stanbury and Morris (35) have recently demonstrated extremely high degradation and excretion rates for this preparation in man. Originally, our observations with this material were based on dosage schedules which involved one subcutaneous injection per rat per day. In view of the possibility that the lack

of activity observed was due to rapid excretion and/or lability of the compound in alkaline solution, a new goiter-prevention assay was run. The 3,3′-diiodo-*dl*-thyronine was dissolved in alcohol/propylene glycol, 10/90, V/V and injected subcutaneously, three times per day per rat, at 4-hour intervals, for fourteen consecutive days. Groups of 5 to 8 rats were employed at each of six dose levels. The total dose per rat per day ranged from 30 μg. to 300 μg. At the completion of this assay it was noted that 3,3′-diiodo-*dl*-thyronine was inactive. Even the highest dose (300 μg./rat /day, equally divided into three injections per day) was unable to produce any sign of reduction in the thiouracil-induced goiters.

The discrepancy between the observations of Roche *et al.* (30), Roche and Michel (31), and Michel (34) versus those of Pitt-Rivers (32), Gemmill (33) and this report for 3,3′-diiodo-*dl*-thyronine (Table 2) suggests a possible inherent difference in assay methods which allows some investigators to find activity (30, 31, 34) while others find none (32, 33 and this report). If this were true one would expect to find similar discrepancies, between the various investigators, for other analogues. However, this does not appear to be the case. A more likely explanation for the discrepancy involves the possibility that the 3,3′-diiodo-thyronine used by Roche *et al.* (30, 31) was contaminated with 3,5,3′-triiodothyronine. Their method of synthesis for the 3,3′-diiodo compound (30) involved the deiodination of 3,5-diiodothyronine to yield 3-monoiodothyronine which was in turn iodinated to yield 3,3′-diiodothyronine. It appears conceivable that some non-deiodinated 3,5-diiodothyronine was present, as a contaminant, during the iodination of the 3-monoiodothyronine, so that not only the desired 3,3′-diiodothyronine but also some 3,5,3′-triiodothyronine would be produced. Since *l*-triiodothyronine is about eight times as active as *l*-thyroxine (Table 1) it could account for virtually all of the activity reported for 3,3′-diiodo-*dl*-thyronine by Roche *et al.* (30) assuming it were present as a contaminant to the extent of 10% by weight as the *l*-isomer. The method of synthesis employed in this laboratory by Meltzer and Stanaback (36), for the preparation of 3,3′-diiodothyronine, precludes the possibility of contamination with 3,5,3′-triiodothyronine. This was accomplished in an intermediate step, by replacing the amino group in α-benzamido-3-amino-4-(4′-methoxyphenoxy)-cinnamic acid with iodine to yield a 3-monoiodo compound free of any 3,5-diiodo contaminant. Direct iodination of the 3-monoiodo compound cannot then introduce an iodine substituent in the 5 position.

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BEHAVIOR OF IODIDE IN ACID SOLUTIONS OF HEMOGLOBIN[1]

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ABSTRACT

Acidification of aqueous solutions of oxyhemoglobin containing I^{131}-labelled inorganic iodide resulted in prompt binding of iodine to the protein, so that an appreciable fraction and quantity no longer behaved as inorganic iodide, as judged by solubility in trichloroacetic acid, dialysis and adsorption by anion-exchange resins. Binding was greatly decreased in the presence of reducing agents (thiosulfate, thiourea, ferrocyanide, ascorbic acid) and of certain halides (thiocyanate and to a lesser degree bromide), but the addition of these substances after binding had occurred failed to release iodide. Most of the bound iodine was released after brief exposure to strong alkali at room temperature. Methemoglobin, reduced hemoglobin, carboxyhemoglobin and hematin were much less effective in the binding reaction than oxyhemoglobin. Bound iodine was found in both the globin and the hemin fractions separated by the acid-acetone method. Consideration of iodine/hemin and iodine/iron ratios led to the conclusion that the iodine in the globin fraction could not be accounted for as ferriheme iodide retained in the globin. The use of trichloroacetic acid as a protein precipitant in solutions containing inorganic I^{131} could result in spuriously high values for PBI^{131} if oxyhemoglobin were present.

Although the nature of the iodine protein combination is not clear, the findings suggest that the binding is a consequence of an oxidative change resulting from the acidification of oxyhemoglobin.

IN USING the trichloroacetic acid procedure for the determination of conversion ratios (PBI^{131}/total I^{131}) in a series of arterial blood serum samples obtained one to three hours after the administration of I^{131} to human subjects, erratic results were occasionally encountered, the values in some samples being appreciably higher than had been expected and than were found in most of the samples in the series. It was noted that the high values were obtained in sera in which hemolysis had occurred, and it was found that if a crystal of sodium thiosulfate was added to such hemolyzed samples before precipitation with trichloroacetic acid, the conversion ratios were comparable to those of non-hemolyzed samples in the series. It appeared likely that the presence of hemoglobin was responsible for these curious effects and a study was made of some of the properties of solutions containing both hemoglobin and iodide.

METHODS

Hemoglobin solutions were prepared from human red blood cells obtained from outdated bank blood. After removal of the plasma by centrifugation, the cells were washed

[1] Presented at the 1958 Meeting of the American Goiter Association.

repeatedly with 0.9% sodium chloride solution and were hemolyzed by being shaken with 1 volume of water and 0.4 volumes of toluene (1). The clear hemoglobin layer was filtered through cotton, the filtrate was dialyzed against several changes of distilled water for several days at 4° C, and the resulting hemoglobin solutions were used for the experiments. Solutions containing approximately 50% methemoglobin were prepared by permitting oxyhemoglobin solutions at pH 5.1 (HCl) to undergo auto-oxidation for 24 hours at room temperature (2). Methemoglobin solutions were also prepared by adding a slight excess of potassium ferricyanide to hemoglobin solutions and then dialyzing exhaustively in tap water and then distilled water. Hemin was prepared from aqueous hemoglobin solutions by the method of Drabkin and Austin (3), and recrystallized from pyridine-chloroform (4).

The acid-acetone procedure of Anson and Mirsky (5) was used to separate hematin from globin. Oxyhemoglobin solutions made .05 to 0.1N in HCl were passed through an anion-exchange resin column and 10 to 15 volumes of acetone containing 1% 1N HCl were added to the effluent to precipitate the globin and dissolve the hemin. Precipitation was carried out in large centrifuge tubes, the supernatant solution was removed by aspiration, and the precipitate washed twice with acetone-HCl and twice with acetone before being dissolved in H_2O to appropriate volume and used for further study. Hemin was precipitated from the chilled acetone supernatant solutions by addition of 1/100 volume of 2N sodium acetate, the precipitate being collected by centrifugation and washed several times with dilute acetic acid. Recovery of hemin was not quantitative.

Radioactivity measurements were made with a well-type scintillation counter, appropriate corrections being applied for variation in sample volume. The radioactive iodine used was carrier-free Na I^{131} in isotonic saline, containing benzylalcohol (0.9%), obtained as sterile solution Sodium Radio-Iodide (I^{131}) Therapeutic from Abbott Laboratories. This material was greatly diluted with distilled water before it was used. In experiments involving trichloroacetic acid (TCA) precipitation of proteins and determination of radioactivity in the precipitate, the dissolved precipitate was counted in the test tube in which the original sample had been measured and in which precipitation and washing had been subsequently performed, so that no transfer of material was required. Precipitation of proteins was made by adding 4 to 8 volumes of ice-cold 10% TCA; ten minutes later the mixture was centrifuged, the supernatant liquid removed by aspiration, the precipitate resuspended in 10% TCA, centrifuged, and the liquid again aspirated. The precipitate was then dissolved in 2N NaOH with mild heating.

In some experiments the anion-exchange resin Amberlite IRA-400 was used to remove inorganic iodide from hemeprotein solutions. A column of resin 60×8 mm. was used, the previously recycled resin being washed with water after having been converted to the acetate or chloride form. One or 2 ml. of solution were added to the column and the effluent (10 to 12 ml.) collected in a graduated centrifuge tube, four or five portions of distilled water being used to wash the column after the sample had run in. This procedure was adequate to recover in the effluent all the heme-colored material which had been placed on the column. Passage of dilute labelled iodide solutions through the column resulted in retention by the column of more than 99% of the I^{131}.

Hemoglobin was determined by the spectrophotometric cyanmethemoglobin method (6) and in some cases by iron determination. Methemoglobin was determined by the method of Heilmeyer (7) and of Evelyn and Malloy (8). Hemin in acetone-HCl solution was estimated spectrophotometrically from the extinction coefficients of Lewis (9). Iron was determined by the method of Lorber (10) as modified by Paul (11).

Paper electrophoresis was carried out between horizontal glass plates on Whatman 3 MM paper (12). Paper chromatograms were made by the ascending method, butanol-acetic acid-water and butanol-ammonia-ethanol being used as solvents (13). Radioautographs were prepared from paper electrophoretograms and chromatograms as previously described (14).

TABLE 1. TCA-PRECIPITABLE I^{131} IN HEMOGLOBIN-IODIDE SOLUTION

Solutions (1.1 ml.) containing hemoglobin (1.44 μM/ml.), carrier-free Na I^{131} and the substances shown were treated with TCA, and the fraction of I^{131} precipitated determined.

Test substance (M/L)	Per cent of I^{131} TCA precipitate
Control	30.6
Perchlorate (.01)	30.6
Thiocyanate (.01)	6.0
Cyanide (.02)	24.3
Thiosulfate (.003)	4.2
Sulfate (.1)	32.0
Ferrocyanide (.001)	7.5

RESULTS

When carrier-free Na I^{131} was added to hemoglobin solutions and the proteins precipitated by TCA, an appreciable portion of the I^{131} present was found in the protein precipitate. The presence of reducing agents (thiosulfate, thiourea, ferrocyanide) and of thiocyanate added to the system prior to precipitation greatly decreased the proportion of I^{131} bound to protein, while perchlorate and cyanide had little effect (Table 1). Binding of I^{131} to the proteins seemed to depend upon acid conditions since passage of neutral hemoglobin-iodide solutions through anion exchange columns yielded virtually no I^{131} in the effluent while prior acidification (0.1N acetic or hydrochloric acid) of the iodide-containing hemoglobin solution resulted in emergence of a considerable quantity of I^{131} from the column with the proteins. The importance of acid in the binding was also shown in the experiments summarized in Table 2. Five ml. aliquots of hemoglobin containing carrier-free Na I^{131} (0.18 μc.) were placed in cellophane dialysis bags which were then immersed in 50 ml. buffer at pH 6.8 or in 0.1N acetic acid; the systems were kept at 4° C with occasional shaking for 72 hours, and at this time the bag contents/medium concentration ratio of I^{131} was 1.49 in the neutral medium, while the corresponding value at acid pH was 8.4. The values for serum and for saline were 1.96 and 1.04 respectively. The increased volume of the bag contents in experiment 2 (column III) suggested that an increase in the number of non-diffusible molecules had occurred, perhaps attributable in part to the cleavage of the hematin from the protein. Since 60% of the I^{131} within the bag was TCA-precipitable in the case of hemoglobin at acid pH, while only 4% of the I^{131} in the serum

TABLE 2. EQUILIBRIUM DIALYSIS OF HEMOGLOBIN-IODIDE MIXTURES

I	II	III Bag		IV Medium		V	VI Bag I^{131}	
Bag (5 ml.)	Medium (50 ml.)	Volume (ml.)	I^{131} (c./m./ml.)	Volume (ml.)	I^{131} (c./m./ml.)	Bag/medium concentration ratio I^{131}	% of total	% TCA precipitable
1 HbO_2 (4% in water, I^{131})	.05M P buffer, pH 6.8	8	4535	47	3035	1.49	20.2	25
2 HbO_2 (4% in water, I^{131})	.10N acetic acid	20	7475	35	889	8.4	82.9	60
3 Normal serum (1:1 in water, I^{131})	.05M P buffer, pH 6.8	7	5785	48	2945	1.96	22.3	4
4 0.9% saline (I^{131})	.05M P buffer, pH 6.8	5	3240	50	3130	1.04	9.4	0

bag was precipitable, it seemed that the concentration gradient in the acid-hemoglobin system was not one predominantly involving iodide.

Since these results suggested that the TCA-precipitability of iodide in hemoglobin solutions depended upon the acidic character of the protein precipitant rather than upon a hemoglobin-iodide interaction prior to precipitation, the remainder of the study was concerned with the characterization of the heme-protein iodide interaction at acid pH.

A. *Iodide concentration.* The effect of varying the concentration of iodide upon the TCA precipitability of the iodine was studied in acidified mixtures of hemoglobin and iodide. In order to make the conditions of exposure to acid solutions more reproducible, the solutions were made up to be .05N in HCl before TCA precipitation. Figure 1 shows the results of such an experiment, the hemoglobin concentration being maintained constant.

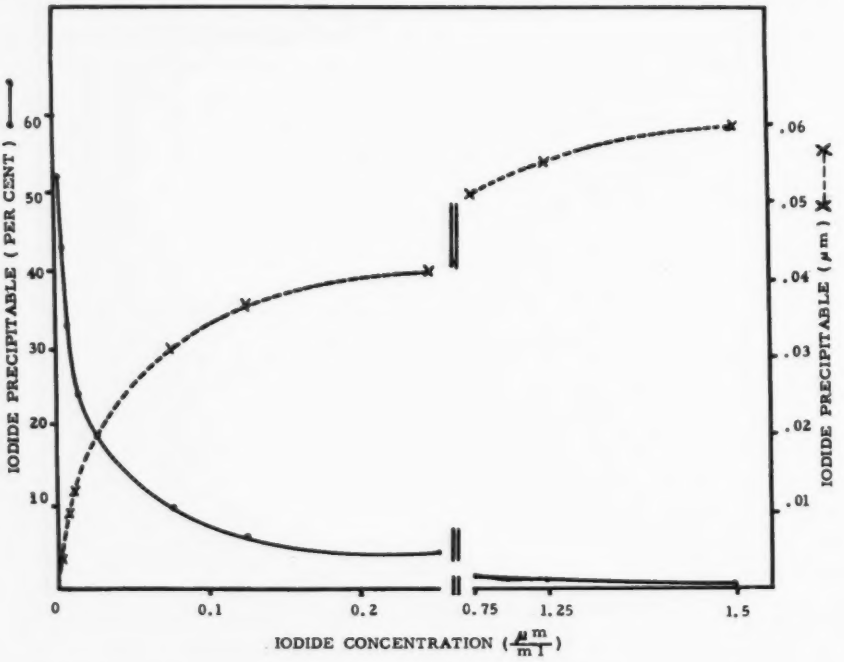


FIG. 1. *Effect of iodide concentration upon iodine precipitability.*

Potassium iodide in amounts ranging from 0 to 10 μM were added to 1 ml. portions of aqueous solutions containing 4.0 μM hemoglobin and 0.15 μc. I^{131}, the final volume being 2 ml.; 2 ml. 0. 1N HCl was then added, and after 10 minutes at room temperature the proteins were precipitated by 10% TCA and the precipitable I^{131} determined. The concentration of iodide was calculated from the amount present in 4 ml. volume.

The unbroken line represents the percentage of the I^{131} present which was precipitated with protein; the broken line indicates the quantity of iodine precipitated (calculated from the known specific activity of iodide in the system). Note change in abscissa scale.

The proportion of the I^{131} which became protein-bound varied from 53% in the absence of added carrier to less than 1% at the highest iodide concentration. However, despite the progressive reduction in the proportion of iodine bound, the quantity of iodide^{127} bound increased progressively as the concentration of stable iodide rose; thus, at iodide concentration of 0.0025 μM/ml. 0.0044 μM iodine was protein-bound, while at iodide concentration 2.5 μM/ml. 0.06 μM was bound.

B. *Hemoglobin concentration.* The effect of varying the concentration of hemoglobin upon the TCA precipitability of iodide in the solution under conditions of constant iodide concentration was studied, and the results appear in Figure 2, which indicates the proportion of the iodide precipitable as the concentration of hemoglobin ranged between 0.007 and 1.4 μM/ml., at two concentrations of iodide (B and C) and also in the absence of added carrier iodide (A). The general shape of these curves was similar to that of Figure 1. At each level of iodide concentration the proportion of the total iodide which was bound increased with increasing hemoglobin concentration. Although the fraction of the iodide bound increased progressively as the concentration of hemoglobin rose, the efficiency of the process tended to decrease since the highest values for the molar ratios of iodine bound/hemoglobin present were observed at the lowest concentrations of hemoglobin. Thus, (curve B, iodide concentration .001 μM/ml.,) at hemoglobin concentration .007 μM/ml. the molar ratio of iodine bound /hemoglobin present was .0031 while at hemoglobin concentration 0.7 μM/ml., this ratio was .00052. As was also seen in Figure 1, at a given hemoglobin concentration a greater fraction of the iodide was precipitable in the more dilute iodide colutions, and the relative positions of curves B′ and C′ confirmed the finding that higher iodide concentration yielded larger quantities of bound iodine. For example, a ten-fold increase of iodide concentration increased the value for the number of moles of iodine bound per mole hemoglobin present at .007 μM hemoglobin/ml. from .0031 to .0129.

C. *Presence of other anions.* The nature and quantity of certain anions in the hemoglobin-iodide solution may modify the proportion of iodide which is precipitable. This was suggested by the observation that if hemoglobin-I^{131} solutions were treated with equivalent concentrations of different acids prior to precipitation with TCA, the percentage of I^{131} precipitable was not the same in each case. For example, pre-treatment of identical aliquots of solutions containing hemoglobin and labelled iodide with 0.1N acetic, hydrochloric, sulfuric and phosphoric acids resulted in the precipitation of 54, 30, 42 and 42% of the I^{131}, respectively, while these values were 42 and 26%, respectively, when 1N acetic and 1N hydrochloric acid were used.Pre-treatment of hemoglobin solutions with sodium chloride-hydrochloric acid mixtures of the same pH but varying ionic strength also suggested an inhibitory effect of the chloride ior.

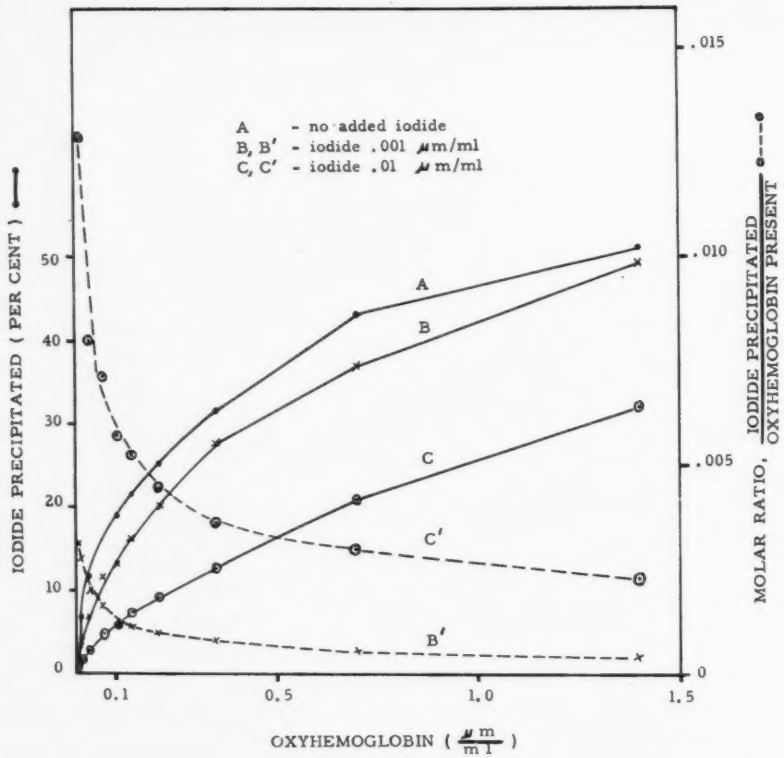


FIG. 2. *Effect of hemoglobin concentration upon iodine precipitability.*

Increasing amounts of hemoglobin ranging from 0.035 to 7.5 μM were added to test tubes containing the same quantity of labelled iodide. Three parallel experiments are shown; tubes of series A contained no added carrier iodide, tubes of series B contained .005, and of series C .05 μM KI^{127} respectively. The total volume of solution was 2.5 ml. To each tube 2.5 ml. 0.1N HCl was then added; one hour later the proteins were precipitated by 10% TCA and precipitable I^{131} determined. The concentrations of iodide and of hemoglobin are calculated from the quantities present in 5 ml. volume.

The percentage of the total I^{131} bound to protein is shown in the unbroken lines (left ordinate), and the values for the moles of iodine precipitable per mole of oxyhemoglobin present are shown in the broken lines (right ordinate), the values in B′ and C′ being calculated from the known specific activities in the corresponding series B and C.

The comparative effects of the presence of chloride, bromide, fluoride, thiocyanate and iodide on the binding of iodine are shown in Figure 3. These ions were added as sodium salts to solutions of hemoglobin and labelled iodide which were then made .05N in HCl and the proteins precipitated with TCA. Under the conditions of the experiment fluoride and chloride had little effect while the presence of bromide caused a fall in the proportion of I^{131} precipitable with protein; at a bromide concentration of

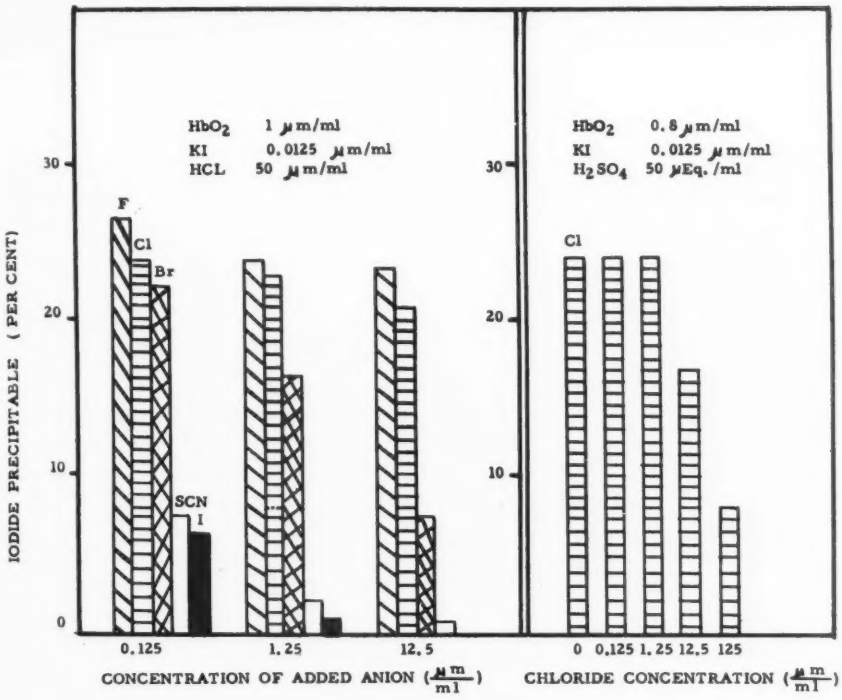


FIG. 3. *Effect of added halides on precipitability of* I^{131}.

Left panel. To a series of tubes containing 4.0 μM HbO_2 and 0.05 μM KI labelled with I^{131} were added fluoride, bromide, chloride, thiocyanate, and iodide, in amounts of 0.5, 5.0, and 50.0 μM as sodium salts, the final volume being 2.0 ml. 2.0 ml. 0.1N HCl was then added to each tube, and after 30 minutes at room temperature, TCA precipitation was performed. The percentage of the total I^{131} precipitated is shown.

Right panel. Experiment similar to A, but the concentration of HbO_2 was smaller, 0.1N H_2SO_4 was used in place of 0.1N HCl, and the concentration of chloride was varied from 0 to 125 μM/ml.

12.5 μM/ml. only one-third as much iodide was precipitable as in the absence of bromide. The inhibitory effect of added chloride (which had probably been masked by the high chloride concentration resulting from the use of hydrochloric acid as the acidifying agent) is apparent in the right panel of Figure 3, where sulfuric acid was used in place of hydrochloric, and a chloride concentration of 12.5 μM/ml. appreciably reduced iodine binding. The great inhibitory effect of thiocyanate upon I^{131} precipitability is shown in Figure 3; the binding of iodine was reduced to 30, 8 and 2% of control values by the three concentrations of thiocyanate tested. The inhibitory effect of thiocyanate is comparable in magnitude to that of iodide itself on the protein-binding of I^{131}, although the iodide ion appeared somewhat more effective. Comparison of the effects of bromide and iodide indi-

cated that iodide contamination of bromide did not account for the inhibitory effects of the latter; since 12.5 μM/ml. of bromide yielded a value for precipitable I^{131} similar to that encountered at 0.125 μM/ml. of added iodide, 1% iodide contamination of bromide would have been required to account for the effects on this basis.

The inhibitory effect of thiocyanate depended not only upon its concentration but also upon the concentration of hemoglobin. This was shown in a study of the effect of thiocyanate upon the TCA precipitability of I^{131} in two hemoglobin-Na I^{131} (carrier-free) systems, the first containing 1.5 Mμ HbO_2/ml. and the second 0.15 μM/ml. In the more concentrated hemoglobin solution, inhibition was found to be 10, 25 and 70% at thiocyanate concentrations of 10^{-4}, 10^{-3}, and 10^{-2} M respectively, while the corresponding values in the more dilute solution were 43%, 58%, and 84%; per cent inhibition was defined as $100 \times (1 - \%I^{131}$ precipitated in presence of thiocyanate/$\%I^{131}$ precipitated in absence of thiocyanate).

D. *Effect of other heme compounds.* Comparison of iodine binding by some heme proteins and by hemin indicated that oxyhemoglobin was by far the most effective substance tested. Table 3 indicates the effects of hematin, oxyhemoglobin and methemoglobin in equimolar concentration in producing TCA insolubility of iodide, under various conditions. An appreciable fraction of the I^{131} was precipitated with hematin only in the absence of carrier iodide; the addition of globin to hemin prior to dissolving in dilute NaOH did not increase the proportion of I^{131} bound. Thiocyanate, thiourea and dithionite are seen to have exerted some inhibitory effect on the carrier-free Na I^{131}-hematin system. Methemoglobin was quite ineffective in binding iodine in acid solution; it is possible that the slightly higher percentage of I^{131} bound in the presence of dithionite than in the control may have been due to oxygenation of reduced hemoglobin. The ineffectiveness of methemoglobin was also apparent in experiments similar to those in Figure 2, in which mixtures of oxyhemoglobin and methemoglobin prepared by auto-oxidation of the former, were used at various concentrations

TABLE 3. TCA-PRECIPITABLE I^{131} IN SOLUTIONS OF HEMATIN, HbO_2, MetHb

The test substances were added to solutions of carrier-free Na I^{131} containing the designated quantities of heme compounds, HCl was added to a final volume of 2.5 ml. and concentration of .05 N, and TCA-precipitable I^{131} determined. Hematin solution was prepared just before use by dissolving recrystallized hemin in 3.1 equivalents of NaOH. MetHb solution was a well-dialyzed sample of ferricyanide-treated hemoglobin.

Substance added (μM)		Hematin, 3.45 μM	Oxyhemoglobin, 3.45 μM	Methemoglobin, 3.45 μM
		% of I^{131} TCA-precipitable		
Control		8.6	56.8	0.9
KI	0.01	1.0	40.0	0.5
KI	0.10	1.0	15.2	0.4
KI	1.00	1.0	3.1	0.4
NaSCN	1.0	2.7	7.1	0.4
Thiourea	1.0	0.7	30.6	0.5
$Na_2S_2O_4$	(excess)	0.5	0.9	1.3

and in different proportions; the curve expressing the relationship between the fraction of I^{131} bound and the HbO_2 concentration of the mixture was very similar to that found in solutions of oxyhemoglobin alone. As indicated in Table 1, ferrocyanide was found to exert a considerable inhibitory effect on the binding; it was therefore essential that solutions of methemoglobin prepared by the ferricyanide method be dialyzed very thoroughly to ensure the absence of both ferri- and ferro-cyanide from the system before using the methemoglobin in experiments on iodine binding, particularly where the effect of the methemoglobin on binding by oxyhemoglobin-containing solutions was studied.

Figure 4A compares equimolar concentrations of oxyhemoglobin, reduced hemoglobin and carboxyhemoglobin with respect to iodine precipitability, and shows the greater proportion bound by oxyhemoglobin. To demonstrate the decreased effectiveness of reduced hemoglobin in this system it was found necessary to equilibrate with nitrogen for 10 to 15 minutes and to exclude air from the system with great care.

In Figure 4B is shown the difference in results observed when acid was added to the oxyhemoglobin solution after, as compared with 10 minutes before, the addition of iodide to the system, the chloride and HCl concentration being the same in the two cases; the experiment indicated again that conversion of oxyhemoglobin to acid hematin in the presence of iodide led to considerable binding of iodine while little bound iodine resulted from the re-acidification in iodide solution of the neutralized acid hematin formed in the absence of iodide.

E. *Iodine in globin and in hemin.* In order to determine whether the bound iodine in the oxyhemoglobin-iodide-HCl system was in the porphyrin or in the globin fraction, the acid-acetone procedure was used as illustrated in Figure 5. The fractions were designated as follows: original solution of HbO_2-iodide^{131}-HCl, A; the anion-exchange column effluent, B; the washed acetone precipitate derived from acetone treatment of the column effluent, G; the acetone supernatant, F; and the hemin derived from the latter, H. The results of 14 experiments such as shown in Figure 5 are summarized in Table 4. These experiments were made under a variety of conditions of temperature, time and concentration of reactants. In 10 experiments in which iodide carrier was used, between 22 and 60% of the iodine in A was not adsorbed by the column (and hence was presumably not iodide) and appeared in B; the values for the TCA-precipitable I^{131} in the A solutions in general were slightly less than those for the I^{131} not retained by the column. An average value of 33% (range 29–43) of the I^{131} emerging from the column was accounted for in the globin precipitate.

The globin precipitate, G, could be redissolved in water and reprecipitated by acetone-HCl with relatively small losses of radioactivity. For example, in one experiment three equal aliquots of the column effluent, B, were subjected to the acid-acetone procedure and acetone-washed fraction

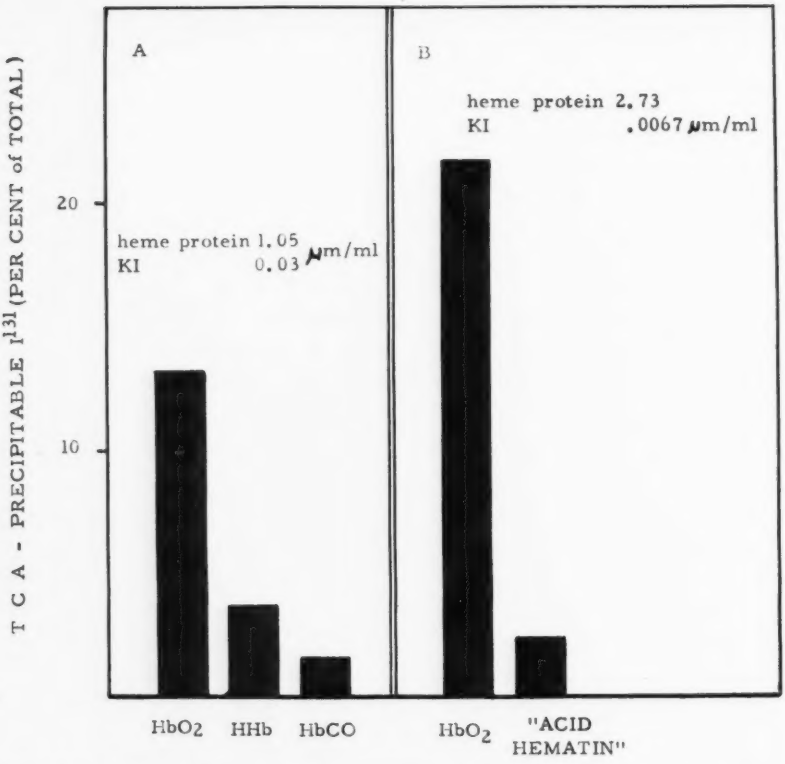


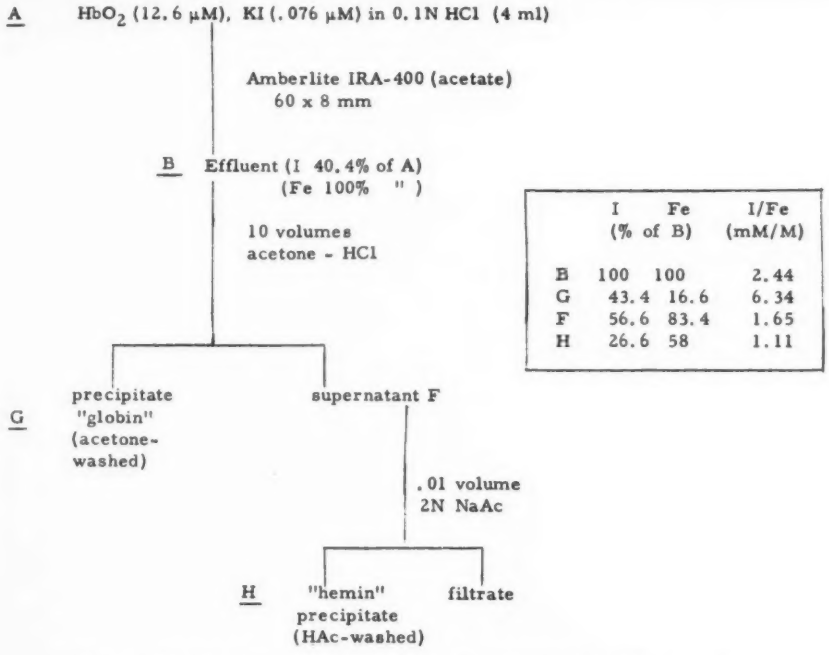
FIG. 4. *TCA precipitable I^{131} in heme protein solutions.*

A. Air, nitrogen, and carbon monoxide were bubbled into solutions containing 6.32 μM HbO_2, 0.182 μM labelled KI and 3 drops octyl alcohol in 4 ml. volume. After 10 minutes and while gas flow continued, 2 ml. 0.1 ml. 1N HCl was injected into each solution via a rubber tube and then TCA precipitation was performed.

B. "Acid hematin" was prepared by adding 0.1 ml. 1N HCl to 1 ml. HbO_2 solution (4.2 μM) and allowing it to stand 10 minutes at room temperature. After neutralization of the mixture with 0.13 ml. 1N NaOH, 0.01 μM labelled KI was added, the solution again made 0.1N in HCl and after 10 minutes, TCA precipitation was done. The HbO_2 control was treated in the same way, except that 0.1 ml. 1N NaCl was used instead of 1N HCl, and water instead of NaOH.

G prepared from each; analysis of one sample was made directly, another after dissolving the precipitate and re-precipitating, and of the third after dissolving and reprecipitating twice; the once, twice and thrice precipitated globin fractions contained 39.6, 35.6 and 32.4% of the I^{131} in the column effluent, respectively, while the corresponding iron values were 14.9, 13.6, and 13.6% of B.

In 6 experiments in which hemin was prepared from the acetone filtrate



	I (% of B)	Fe	I/Fe (mM/M)
B	100	100	2.44
G	43.4	16.6	6.34
F	56.6	83.4	1.65
H	26.6	58	1.11

FIG. 5. Distribution of iodine and iron in acetone-soluble and insoluble fractions of acidified oxyhemoglobin.

the washed, isolated (but not recrystallized) hemin accounted for 42.2% (range 25–57) of the I^{131} in F.

The presence of reducing agents (thiourea, ascorbic acid) in solution A prior to acidification resulted in almost complete removal of I^{131} by the resin column, so that little I^{131} appeared in B, a result comparable to the diminution in the fraction of iodine precipitable by TCA under similar circumstances. In an experiment identical with experiment 4 in Table 4, except that thiourea 40 μM/ml. was present in the HbO_2-iodide solution before acidification, only 3.2% of the I^{131} in A appeared in B, and G contained only 4.4% of the RaI in B, in contrast to corresponding values of 22 and 29% in the absence of thiourea.

The "globin" (G) prepared by the procedure described had a brownish color, and its concentrated solutions were yellow; in alkaline solution and in the presence of a reducing agent (dithionite), the absorption spectrum of G was similar to that of globin hemochromogen but the maxima were shifted 4–5 mμ toward the shorter wavelengths (maxima at 554 and 523 mμ); the material exhibited peroxidase activity by the benzidine test; and when G was prepared from hemoglobin solution to which a reducing agent had been added prior to acidification, and binding of iodine had been

TABLE 4. IODINE AND IRON IN "GLOBIN" PREPARED FROM RESIN-TREATED ACID SOLUTIONS OF OXYHEMOGLOBIN AND IODIDE

A, B, and G refer to the fractions so designated in Figure 4.

Expt.	HbO_2	KI	HCl	I^{131} in column effluent	I^{131} in "globin" (G)	Fe in globin	I^{131}/Fe*
	μM/ml.	μM/ml.	M/L	(% of A)	(% of B)	(% of B)	
1	3.15	0.019	0.1	40	43	17	2.53
2	2.63	0.018	0.1	30	29	16	1.81
3	2.58	0.019	0.05	33	36	14	2.57
4	3.0	0.019	0.055	22	29	18	1.61
5	5.3	0.011	0.09	28	29	—	—
6	3.1	0.02	0.10	22	31	12	2.58
7	2.32	0.003	0.09	30	27	—	—
8	2.7	0.004	0.17	24	30	11	2.73
9	2.0	0.0044	0.05	38	41	16	2.56
10	2.2	0.005	0.06	60	35	—	—
					av.: 33	av.: 14.9	I^{131}/Fe =2.34 av.
11	2.26	no carrier	0.05	59	34	—	—
12	3.05	no carrier	0.10	60	42	—	—
13	2.9	no carrier	0.10	78	44	18	2.44
14	2.63	no carrier	0.10	76	45	19	2.36
					av.: 41.3	av.: 18.5	I^{131}/Fe =2.40 av.

* $I^{131}/Fe = \frac{I^{131} \text{ in G(\% of B)}}{Fe \text{ in G(\% of B)}}$.

largely prevented, the acetone precipitate was much lighter in color. Since hemin (ferriheme chloride) can be readily converted to iodohemin (i.e. ferriheme iodide) (15), and since it has been claimed (9, 16) that acidification of oxyhemoglobin and subsequent extraction with acetone or ether liberates only part of the hemin from the protein unless a reducing agent is present, the possibility was considered, in view of the aforementioned observations suggesting that the "globin" was not free of hematin, that the I^{131} in the globin fraction was actually bound to hematin not separated from the globin by the acid-acetone procedure, particularly since some I^{131} was found in the hemin isolated from the acetone soluble fraction in these experiments. This possibility was explored in two ways: (1) by determination of iron in the globin precipitate as well as in A and B, and by comparison of the iron and iodine content of these fractions, and (2) by studying the hemoglobin-iodide^{131} system at various values of pH and comparing the fraction of I^{131} and of hemin in the acetone soluble and insoluble fractions.

(1) Iron analysis indicated that usually all the iron in A was recovered in the column effluent B, while an average of 14.9% of the effluent iron was present in fraction G (Table 4). The iron analyses were in good general agreement with spectrophotometrically determined values for hemin in the acetone filtrate. The value for the fraction of I^{131} present in G was always greater than the corresponding iron value, the ratio being 2.3 (Table 4).

Since the I^{131}/Fe ratio was always higher in the "globin" than in the acetone supernatant, in the column effluent, or in the hemin (see insert, Fig. 5), it seemed unlikely that the I^{131} bound to the globin fraction could be accounted for on the basis of ferriheme iodide adherent to the acetone precipitate.

(2) Parallel observations were made of the effect of varying the pH of HbO_2-I^{131}-HCl solutions upon I^{131} binding and splitting of hemin from the protein. These experiments were modelled after those of Lewis (9) and the results are shown in Figure 6. Solutions of HbO_2 and I^{131} of constant ionic strength were maintained at pH values ranging from 6.5 to 2.0 and aliquots of each solution were used to determine the fraction of the total I^{131} bound (by the anion-exchange resin method), the fraction precipitable by TCA and by acetone, and the proportion of the total hemin soluble in acetone. As the pH decreased, a larger fraction of the I^{131} became bound (curve II) the binding becoming appreciable at pH 4.5 and continuing to rise to pH 2.0. In general the curve describing TCA-precipitability (IV) paralleled curve II. The plateau of curve IV between pH 6.5 and 4.8 could represent the acidifying effect of the precipitant itself. It was of interest to find that maintenance of the solutions at pH 4 to 2 with mineral acid resulted in progressively higher values for the TCA precipitable I^{131} possibly because acidification with TCA alone may not have permitted completion of the reaction before the protein was thrown out of solution. The curve representing I^{131} bound was similar to that of hemin split from the protein (curve V), suggesting that the formation of acid hematin and the binding of iodine occurred *pari passu*. However, below pH 3.5, 75–90% of the hemin was split from the protein while 25–30% of the total I^{131} present was in the acetone precipitate (curve I). When the I^{131} in the acetone precipitate was expressed as a fraction of the bound I^{121} (curve III), it was apparent that in the pH range 2–3 45% of the bound I^{131} was in the protein precipitate, which at most contained only 15% of the hemin. The increase in bound I^{131} despite achievement of a plateau for the percentage of acetone-precipitable I^{131} suggested that at the lower pH values ferriheme itself bound iodide, and this view was consistent with the finding that TCA-precipitable I^{131} always exceeded the acetone-precipitable I^{131}. Figure 6 also shows that the presence of a reducing agent (ascorbic acid) (points marked A) not only sharply decreased the binding of I^{131} but also permitted quantitative cleavage of hematin from the acidified oxyhemoglobin. The results suggest that the binding of I^{131} by the heme protein paralleled the formation of acid hematin, but quantitative considerations suggest, as do the I/Fe ratios in globin, that iodohemin adherent to the protein did not account for the I^{131} bound to globin.

A few observations have been made concerning some of the properties of the bound iodine in the globin precipitates and column effluents of acidified hemoglobin-iodide^{131} solutions. TCA precipitation of these solutions

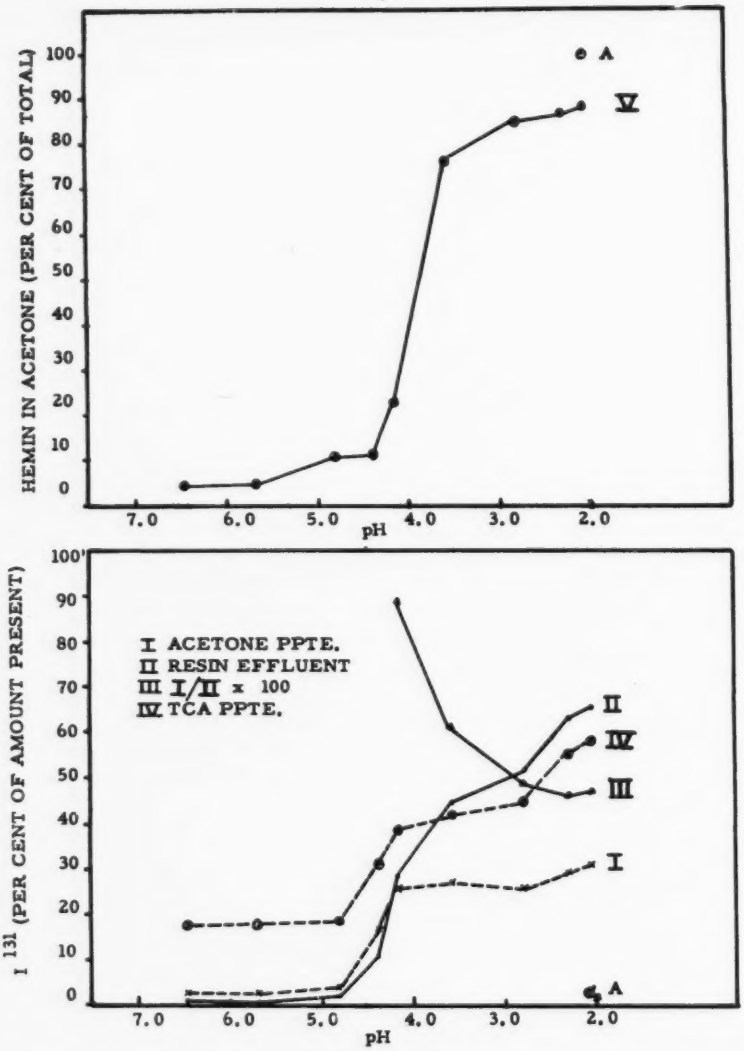


FIG. 6. *Effect of pH on I^{131} bound to protein and on separation of hemin from globin.*

Solutions of oxyhemoglobin (0.77 μM/ml.) containing carrier-free NaI^{131} were adjusted to various pH, ionic strength being maintained constant (.033) by appropriate NaCl-HCl mixtures. Aliquots were used to obtain the data shown. Curve I represents the percentage of the total I^{131} precipitable by acetone at each pH, curve II the percentage not adsorbed by anion-exchange resin, curve IV the percentage precipitable by TCA. Curve III is derived from I and II and indicates the fraction of bound I^{131} present in the acetone precipitate. Curve V (upper panel) shows the percentage of the total hemin which was present in the acetone solutions.

The points marked A are values for a solution to which ascorbic acid was added prior to acidification to pH 2.06.

showed 70–80% of the I^{131} in both B and G to be precipitable and similar values were found on permitting B and G to pass through anion-exchange columns, 70–80% of the I^{131} present emerging from the column. Dialysis of solutions of the labelled acetone precipitate (within the bag) against 10 volumes of distilled water resulted in bag/medium I^{131} concentration ratios of 15 to 25. After prolonged dialysis of labelled G solutions the I^{131} within the bag was still not completely TCA precipitable. These results suggested some instability in neutral and acid solutions of the linkage by which iodine was bound to protein.

The addition of large quantities of iodide, thiocyanate or reducing substances to labelled B or G solutions did not change the proportion of I^{131} that was TCA-precipitable. A brief exposure to 0.5N NaOH, at room temperature, however, decreased the percentage of I^{131} precipitable by TCA or not retained by anion-exchange columns to 10–20% of the values prior to NaOH exposure.

Chromatographic studies of B and G after pancreatic enzyme hydrolysis failed to reveal the presence of labelled mono- or diiodotyrosine, thyroxine or triiodothyronine. Chromatograms (butanol-acetic acid-water) of unhydrolyzed B showed a benzidine-positive, brown spot near the solvent front which contained I^{131} and this was tentatively identified as hemin; most of the radioactivity and pigmented material remained at the origin, but a faint radioactive zone in the iodide region was also usually detectable. Paper electrophoresis of G at pH 5.5 for two hours revealed most of the I^{131} to be at the origin, as was the protein but some I^{131} had the mobility of iodide. Pre-treatment of G with strong alkali and partial neutralization prior to electrophoresis resulted in intensification of the iodide zone and considerable diminution of the activity at the origin. More prolonged electrophoresis at acid pH (18 hours) resulted in a long cathode-migrating smear in which protein (Durrum reagent), peroxidase activity (benzidine) and radioactivity were superimposable. In general, the chromatographic and electrophoretic results confirmed the impression gained from other experiments that the I^{131} was present in both hemin and globin, and that alkali released iodide from the bound form.

DISCUSSION

These experiments indicate that appreciable quantities of iodine may become bound—i.e. may no longer behave as inorganic iodide, as judged by TCA-precipitability, dialyzability and behavior toward anion-exchange resins—when iodide-containing solutions of oxyhemoglobin are acidified. The iodine was found in both the protein and the hemin, the proportion and amount varying with conditions of pH, concentrations of reactants, ionic strength, and other factors. Although some hemin or a hemin derivative was present in the globin fraction prepared under conditions leading to iodine binding, quantitative considerations failed to support the idea that all the protein-bound iodine was iodohemin. Using the observations

that the "globin" contained, on the average, 2.3 times as much of the total bound iodine as of the iron in the acid-iodide-oxyhemoglobin system, that 15% of the total hemin was retained in the "globin," that only 80% of the I^{131} in G was TCA precipitable, and assuming that all the I^{131} in the acetone filtrate was hemin-I^{131}, one may calculate that only 45% of the globin iodine could be accounted for as iodohemin. This calculation is based on the assumption that all the heme molecules in hemoglobin are equal in their iodine-combining capacity. There is some evidence that one of the hemes in methemoglobin may be dissimilar to its fellows in its behavior toward acid cleavage (9), and it is perhaps possible that the hemin firmly bound to the globin could have a larger iodine/iron ratio than existed in the hemin that was extractable by acetone.

The effect of bromide chloride and particularly of thiocyanate in decreasing the binding of iodine could in part be related to competition between these anions and iodide for the formation of halide derivatives of hematin, but the observation that only a fraction of the bound iodine was accounted for as iodohemin suggests that this does not completely explain the inhibitory mechanism. The failure of cyanide appreciably to affect iodine binding in this system may be related to the very limited dissociation of HCN at the low pH of the reaction mixture.

The reasons for the variation among the values listed in Table 4 for the fraction of the total iodine found in the acetone precipitate is not entirely clear. The experiments differed in concentration of reactants, pH and ionic strength, which are factors known to influence the results. It is also possible that the temperature at which acidification occurred and the time elapsing between acidification and precipitation, factors which were not controlled in these experiments, could be important. The inconstancy of the iodine composition of the globin-iodine compound, as reflected in the diminishing iodine content resulting from repeated precipitations, is another factor that might be responsible for variation.

The curves expressing the relationship between the quantity of iodine bound and both the iodide concentration and the hemoglobin concentration (Figs. 1 and 2) appear to be of exponential nature. Re-plotting the data of curves B′ and C′ in Fig. 2 as a log-log graph indicated a straight line relationship between log (quantity of iodine bound) and log (hemoglobin concentration) at each iodide concentration; a similar graphical method applied to the data of Figure 1, i.e. plotting the log (quantity of iodine bound) against log (concentration of iodide) did not yield a straight line, and it is therefore likely that an equation of the Freundlich adsorption isotherm type does not describe the relationship between these quantities.

Although hemin (via alkaline hematin) could combine with trace quantities of iodide under the conditions of these experiments, of all the heme compounds tested (HbO_2, HHb, HbCO, MetHb, and hemin) oxyhemoglobin was by far the most effective in the iodine-binding system. These ob-

servations together with the failure of acid hematin to combine appreciably with iodide, suggested that (ferrous) heme attached to globin and combined with oxygen was required for maximal iodine binding consequent to acidification. It is known that certain reducing substances (ascorbic acid, epinephrine) cannot be accurately determined in hemolyzed sera deproteinized by trichloroacetic acid, and it has been suggested that the liberation of "active oxygen" which occurs when oxyhemoglobin is acidified leads to oxidation not only of the ferrous iron but also of oxidizable substances in the solution, including globin itself (17). Acidification of oxyhemoglobin fails to free globin entirely of hemin, and some of the pigment is very firmly bound to the protein, perhaps by a reactive group formed as a consequence of globin oxidation (16, 18); if a reducing agent is present or if oxyhemoglobin is converted to a non-oxygen-containing heme protein prior to acidification, the globin can be almost completely freed of heme (9, 16). In addition to this mechanism of oxidation by oxygen released from combination, it has also been suggested that hydrogen peroxide may be formed when oxyhemoglobin is acidified, and the peroxide may oxidize appropriate substances that may be present, hematin acting as peroxidase (19). The importance of the oxidative change in effecting the binding of iodine was implied not only by the effectiveness of oxyhemoglobin as compared with other heme proteins but by the effect of reducing agents in decreasing the binding. The studies of Lemberg (19) of the oxidation of ascorbic acid by solutions of oxyhemoglobin undergoing acidification emphasized the rapidity both of the reaction and of the disappearance of the oxidizing system after acidification, features which were characteristic also of the oxyhemoglobin-iodide-acid system. It is not clear from the data available whether the inhibition of iodine binding by reducing agents such as ferrocyanide or thiosulfate is attributable to the presence of an oxidizable substance which may spare iodide or to a direct reduction by these substances of an oxidized form of iodine.

The nature of the protein-bound iodine is not known. The preliminary chromatographic and electrophoretic studies have indicated the presence of labelled hemin, labelled protein, and, after hydrolysis, inorganic iodide. The alkali-lability of the binding, its greater stability in acid, the nondialyzable character of the bound iodine and the apparent lack of effect of reducing agents upon the compound once formed are striking features. Instability in alkali is not characteristic of iodotyrosines and no evidence of these compounds was detected. If oxidation of iodide to iodine by active oxygen were the mechanism of iodination, the low pH at which the iodination occurred might conceivably prevent iodination of tyrosine residues, since alkaline conditions facilitate this reaction (20), which proceeds very slowly below pH 3 (21). An iodination that could occur at acid pH is the formation of a sulfenyl iodide, by the reaction between iodine and a thiol group (20). Although sulfenyl iodides are considered unstable, evidence

has recently been presented indicating the formation and persistence of this grouping as a result of the reaction of iodine with tobacco mosaic virus (22).

The fact that even at very low iodide^{127} concentrations seldom was more than 50 to 60% of the available iodine bound suggested that the sequence might possibly be:

$$2I^- \xrightarrow{\text{oxidation}} I_2 \xrightarrow{+RH} HI + RI$$

Until characterization of the products has been achieved, such an idea is, of course, speculative.

It is believed from quantitative considerations mentioned above, that although the iodine-globin compound was not free of hemin (or a derivative), one may conclude that the iodine-protein binding may occur as a consequence of the same oxidation effects which produce adherence of hemin to the protein rather than that adherent iodohemin is necessarily the form in which iodine occurs in the protein.

Some aspects of the iodine-protein combination bear a superficial resemblance to iodine metabolism of the thyroid gland; in particular the capacity of the oxyhemoglobin solution to abstract iodine from a surrounding medium at low pH (Table 2), the considerable inhibitory effects of thiocyanate and thiourea, and the dependence upon "oxidative conditions." Despite the unphysiological pH at which this reaction occurs and other obvious differences from either thyroidal "trapping" or organic binding of iodine, one wonders whether conditions might not exist under which iron porphyrin proteins such as peroxidase or the cytochromes might bind iodine and serve as an iodide transport mechanism.

The iodine binding that can occur when oxyhemoglobin undergoes conversion to acid hematin, together with the observations on hemolyzed sera mentioned in the introductory statements, suggested that the use of TCA as a protein precipitant might yield spuriously high values for PBI^{131} in systems containing oxyhemoglobin and iodide^{131}. Incubation of homogenates of blood-containing organs, such as spleen, with I^{131} was sometimes associated with the occurrence of appreciable I^{131} in the TCA-precipitate of such a system (23). The effect of the presence of various concentrations of hemoglobin in serum containing iodide^{131} upon the conversion ratio as determined by the TCA method is seen in Figure 7. At a serum hemoglobin concentration of 500 mg.%, 10% of the I^{131} was precipitable and this was almost completely prevented by addition of thiourea prior to precipitation. Hemolysis of this degree might therefore cause an error of 10 to 40% in serum conversion ratios of euthyroid subjects. Serum seemed to exert some inhibitory effect on the TCA-precipitability of iodine in hemoglobin solutions as shown by the higher values for TCA-precipitable I^{131} in the absence of serum (Fig. 7); possible causes of this inhibition, which would

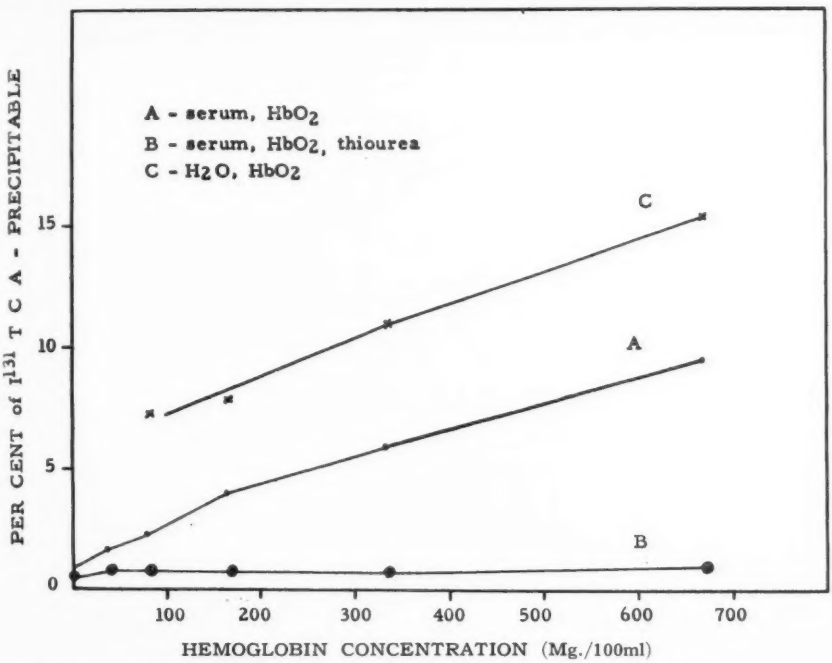


FIG. 7. *TCA precipitability of serum iodide-I^{131}; effect of added hemoglobin.*
Aliquots of serum (0.5 ml.) containing carrier-free NaI^{131} were adjusted to different hemoglobin concentrations, the total volume being 1.5 ml., and proteins were precipitated by TCA (A). B is same as A save for the presence of thiourea (3.3 mgm./ml.) added prior to TCA. C represents hemoglobin and NaI^{131} in absence of serum.

decrease the precipitation error that might otherwise occur, include the presence of reducing substances, inorganic iodide and other halides.

Although analyses of TCA-precipitable I^{127} in serum containing hemoglobin were not made, one might anticipate from the data shown in Figures 1, 2 and 7 that increased values for PBI^{127} might be encountered in serum samples containing a high concentration of hemoglobin, particularly if the serum $iodide^{127}$ concentration were also high.

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COMPARISON OF THYROIDAL AND GASTRIC IODIDE PUMPS IN RATS[1]

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ABSTRACT

Low iodine intake activated the thyroidal iodide pump of rats but had no effect on the gastric pump. Thyroid/serum (T/S) and gastric juice/serum (J/S) radioiodide concentration ratios were similar in rats receiving adequate amounts of dietary iodine. When graded doses of NaI^{127} or $NaClO_4$ were injected, the T/S was more markedly depressed at any dose level used than was the J/S. In rats on a diet with adequate iodine content the capacity of the rat's stomach juice for "pumped" iodide was found to be higher than that of the thyroid. Analysis of the findings suggests that the gastric iodide pump differs from its thyroidal counterpart a) by not being responsive to TSH and to low serum iodide levels, and b) by having a smaller affinity for iodide.

SINCE transport of iodide across the stomach wall is not followed by incorporation of the iodide into thyroglobulin, the process can be studied without recourse to the methods of "chemical dissection" used in investigating the thyroidal iodide pump. It would be convenient if the gastric and the thyroidal iodide pump were in every respect comparable, and results obtained regarding the nature, kinetics and regulation of the former could be held valid for the latter as well. Indeed, gastric and thyroidal iodide transport do show similarities: a) at physiologic serum iodide levels stomach juice/serum and thyroid/serum iodide concentration gradients of rats are approximately the same (vide infra); b) both mechanisms are inhibited by thiocyanate (unpublished observations) and perchlorate (1) in rats, as they are in other species (2, 3, 4). On the other hand thyrotrophin (TSH), to which the thyroidal iodide pump is exquisitely sensitive (5, 6, 7), has no unequivocal effect on gastric iodide transport (8). This paper points out further differences between the gastric and the thyroidal iodide pump of rats with regard to their responses to a) low iodine intake, b) elevation of serum iodide levels and c) graded doses of perchlorate.

MATERIALS AND METHODS

Young adult male Holtzman rats were used. For three weeks they were fed either a Remington diet (Low Iodine Test Diet, Nutritional Biochemicals Corporation, Cleveland, Ohio) or the same diet fortified with 1 mg. of iodine (as NaI) per kg. They were fasted for 24 hours before the experimental procedures, which were the following: a) ligation of pylorus and cardia, and either nephrectomy or, in most instances, tying off

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of all the structures in the renal hilum performed in order to obviate the influence of renal excretion on the equilibration of I^{131} (9); the operations were carried out under ether anesthesia; b) subcutaneous injection of 12 mg. of propylthiouracil (PTU) in 0.6 ml. of alkaline solution immediately after closure of the abdominal incision, and c) subcutaneous injection of a tracer dose of I^{131} one-half hour later. I^{131} was given in one ml. of isotonic (154 mM/l.) NaCl solution, or with graded doses of NaI^{127} or $NaClO_4$. Both NaI^{127} and $NaClO_4$ were prepared in isotonic solutions. When the dose given was less than the NaI^{127} or $NaClO_4$ contained in one ml. of this solution, the injected volume was made up to one ml. with isotonic NaCl. The rats which received the highest dose of NaI^{127} were injected with five ml. of the stock solution. In all cases of NaI^{127} administration the tracer dose of I^{131} was thoroughly mixed with the solution of NaI^{127} given, and specific activity was determined. Four hours after the injection of I^{131} the rats were killed. Blood was obtained from the abdominal aorta, the gastric contents were collected, and the thyroid was dissected out and weighed on a Roller-Smith balance. One-tenth ml. of serum and of the supernatant of the centrifuged gastric contents, as well as the thyroid, were prepared for radioactivity determination by the method of VanderLaan and Greer (5).

The following abbreviations will be used:

$T = I^{131}$ (or labelled I^{127}) per 100 mg. thyroid
$S = I^{131}$ (or labelled I^{127}) per 0.1 ml. serum
$J = I^{131}$ (or labelled I^{127}) per 0.1 ml. gastric juice

Several pilot experiments were carried out on rats fed the fortified Remington diet or Rockland pellets for three weeks, subjected to the operative procedures listed above, and injected with a tracer dose of I^{131} one-half hour after the administration of 12 mg. of PTU. a) Since some of our rats received NaCl while others did not, the effect of this salt on gastric and thyroidal iodide transport was examined. As much as 5 ml. of isotonic NaCl given with the I^{131} had no effect on gastric or thyroidal iodide concentrating ability. Controls received 5 ml. of isotonic $Na_2S_2O_3$. The J/S in these groups was 31.8 ± 2.6 (S.E.)(9 rats) and 38.9 ± 4.0 (10 rats) respectively. b) J/S ratios 6 hours after the administration of I^{131} were no greater than at 4 hours. Numerous earlier observations have indicated that the T/S ratio becomes stable within an hour or less after the injection of I^{131}. The J/S and T/S values to be presented can therefore be regarded as equilibrium concentration ratios. c) Attempts were made to correlate the amount of gastric juice secreted in 4 hours with the J/S, since the volume of the supernatant of the centrifuged gastric juice from animals used in this study varied from only a little over 0.1 ml. to 5–6 ml. No correlation between the J/S and the amount of gastric juice secreted could be established. Because of this and the lack of systematic variation in the amount of gastric juice among groups receiving different forms of treatment, rate of gastric juice secretion was not regarded as a heteropoietic factor in this study. d) In four rats, 92 ± 1.5 (S.E.)% of the I^{131} of the gastric juice (four hours after the injection of tracer amounts) was found in the supernatant when 1 part of centrifuged stomach juice was precipitated with 9 parts of 10% trichloroacetic acid this agrees with chromatographic findings (10) which showed 90–95% of the I^{131} in gastric juice to be in the form of iodide. In six rats 83 ± 5.2% of serum I^{131} was not precipitated by trichloroacetic acid four hours after the injection of the tracer. This was presumably not due to the presence of hormonal I^{131} in the serum, since PTU had been given in sufficiently large amounts to prevent thyroid hormone formation. Ingbar and Freinkel (11) have also found that about 20% of I^{131} added to human serum is not dialyzable. Binding of I^{131} in serum and gastric juice was neglected in the interpretation of our results. It is unlikely that the error thus introduced exceeded 10% in any group.

For the computation of the concentration of "pumped" I^{127} in thyroid and gastric

TABLE 1. THYROID/SERUM (T/S) AND GASTRIC JUICE/SERUM (J/S) I^{131} CONCENTRATION RATIOS IN RATS FED A REMINGTON DIET OR THE SAME REGIMEN FORTIFIED WITH IODINE (1 MG./KG.)

Diet	No. rats	Body wt., gm.	Thyroid wt., mg.	T/S	J/S
Remington	8	236 ± 4*	17.2 ± 1.4	151.4 ± 10.1	30.3 ± 6.0
Remington + I	9	247 ± 7	11.5 ± 0.5	31.1 ± 4.4	36.4 ± 4.3

* Mean ± standard error.

juice the I^{127} which had entered by diffusion was estimated as $\frac{1}{3}$ and $\frac{3}{4}$, respectively, of the serum I^{127} concentration (1), and was subtracted from the total concentration of I^{127} (Table 2, Fig. 1).

RESULTS

Table 1 shows that the T/S was greatly increased in rats on a low iodine diet, which confirms earlier observations (12, 13), but that the J/S in the same rats was no greater than in rats which had received adequate amounts of iodine.

When I^{131} was given with graded doses of I^{127} (Table 2), the T/S was more readily depressed than the J/S. Figure 1 shows that in the gastric juice, as in the thyroid, the concentration of "pumped" I^{127} rose and then tended to level off as serum I^{127} was elevated; the plateau, which indicates the capacity for "pumped" I^{127}, was more than five times as high for the gastric juice as for the thyroid. The highest concentration of "pumped" I^{127} in the gastric juice of any animal in this study was 350 μg./0.1 ml., compared with 46 μg./100 mg. in the thyroid which had taken up the largest amount of iodide. Whereas the average J/T ratio was close to 1 when only a tracer dose of I^{131} was given to rats fed the Remington diet with added iodine, this value rose to 5.5 when serum I^{127} was increased to about 20 μg./0.1 ml., and declined again when serum I^{127} levels were further elevated (Fig. 2). This decrease at very high serum I^{127} levels was a predictable finding. If iodine entered both gastric juice and thyroid by diffusion only, a J/T of about 2.3 (see broken line in Fig. 2) would be expected on the basis of studies with large doses of perchlorate (1). The J/T would tend to approach this value as serum I^{127} levels are raised so high as to flood the thyroidal and gastric pumps and eventually render diffusion of iodide into thyroid and gastric juice preponderant over active transport.

TABLE 2. EFFECT OF NaI^{127} ON THYROIDAL AND GASTRIC IODIDE PUMPS IN RATS FED A REMINGTON DIET WITH IODINE (1 MG./KG.) ADDED

No. rats	Body wt., gm.	Dose of NaI^{127} μM	Serum I^{127}(S), μg./0.1 ml.	Thyroidal "pumped" I^{127} (T minus S/3) μg./100 mg.	"Pumped" I^{127} in gastric juice (J minus 3S/4), μg./0.1 ml.	T/S	J/S
9	247 ± 7*	0	—	—	—	31.1 ± 4.4	36.4 ± 4.3
7	254 ± 8	6.16	0.71 ± 0.11	14.3 ± 1.5	18.1 ± 3.5	21.8 ± 2.6	31.5 ± 7.9
6	242 ± 11	30.8	3.5 ± 0.33	15.9 ± 2.1	56.7 ± 13.4	5.5 ± 1.3	16.5 ± 2.6
8	240 ± 9	154.0	22.4 ± 1.4	23.9 ± 3.3	157.1 ± 34.5	1.4 ± 0.35	7.7 ± 1.3
5	228 ± 15	770.0	132.2 ± 2.1	36.5 ± 5.6	178.7 ± 52.0	0.62 ± 0.12	2.1 ± 0.4

* Mean ± standard error.

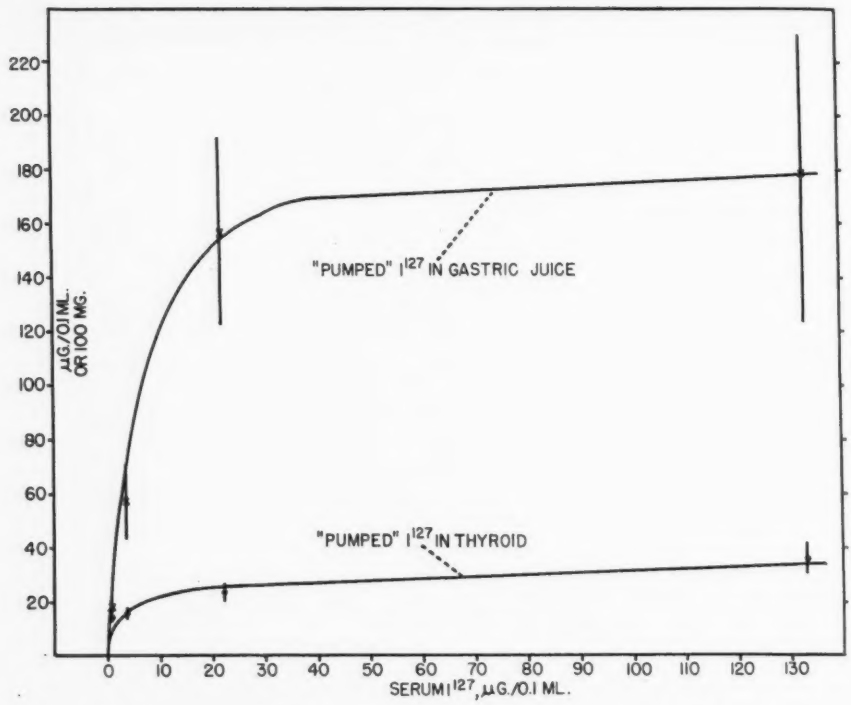

FIG. 1. Influence of serum iodine levels on the concentration of iodine in thyroid and gastric juice. Vertical lines: standard errors of the mean.

With graded doses of $NaClO_4$ similar results were obtained as with NaI^{127}: the highest dose lowered the J/S to 46% and the T/S to 11% of the control values (Table 3).

DISCUSSION

The rats fed a low iodine diet were probably secreting increased amounts of TSH, since their thyroid weights were significantly higher than those of the controls (Table 1). The high T/S of iodine deficient rats has been interpreted as the response of a sensitized iodide pump to normal or elevated levels of TSH (12, 13, 14). The failure of the gastric iodide pump to respond to iodine deficiency in part undoubtedly reflects the lack of effect of TSH on this mechanism, which was also evident in hypophysectomized rats given exogenous TSH (8). Since the gastric iodide pump did not even react to low iodine intake with the moderate activation observed in the case of the thyroidal pump of hypophysectomized rats fed a Remington diet for 19–20 days (13), the gastric wall apparently lacks an intrinsic regulator of its iodide pump like that postulated to exist in the thyroid (12, 13, 14).

Although the T/S and the J/S were approximately equal in rats fed a

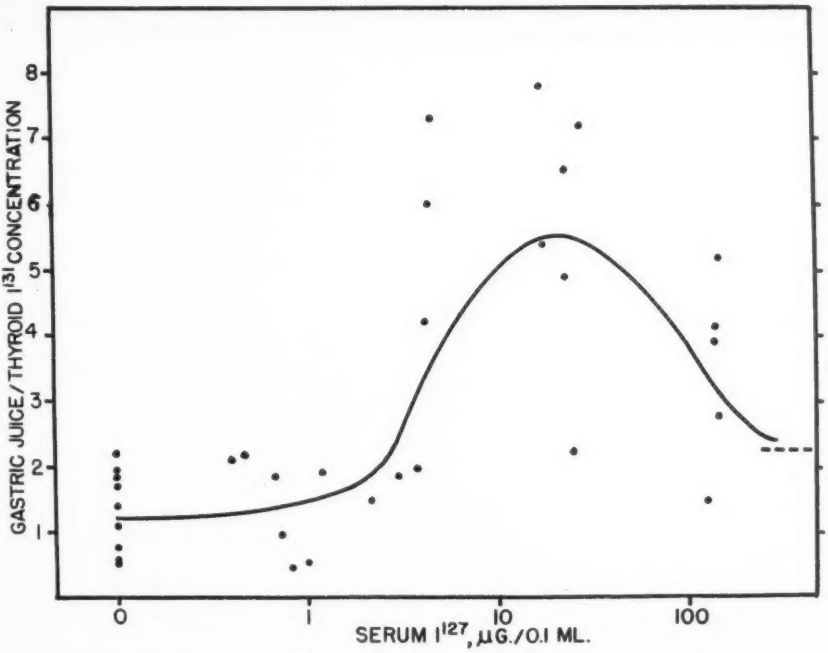

FIG. 2. Influence of serum iodine levels on the gastric juice/thyroid radioiodine concentration ratio. Each point stands for one rat. The line is drawn through the mean values observed in the groups of rats which were injected with NaI^{127} at the dose levels shown in Table 2.

diet containing adequate amounts of iodine and injected only with a tracer dose of I^{131}, loading with I^{127} showed that gastric juice has a much higher capacity for iodide than does the thyroid (Table 2, Fig. 1). This higher capacity, of course, may be entirely due to the fact that a known site of iodide concentration in the stomach, its secretion, was compared with the entire thyroid, which undoubtedly includes compartments in which iodide is not concentrated. An increase in the capacity of the thyroid for iodide is elicited by thyrotrophic stimulation, but this is accompanied by a commensurate increase of the T/S at physiologic serum iodide levels (15, 16). The activated thyroid thereby differs from the stomach. In the analysis of

TABLE 3. EFFECT OF $NaClO_4$ ON THYROIDAL AND GASTRIC IODIDE PUMPS IN RATS FED A REMINGTON DIET WITH IODINE (1 MG./KG.) ADDED

No. rats	Body wt., gm.	Dose of $NaClO_4$, μM	T/S	J/S
9	247 ± 7*	0	31.1 ± 4.4	36.4 ± 4.3
6	252 ± 4	0.308	17.4 ± 2.6	27.7 ± 9.1
6	242 ± 5	3.08	3.3 ± 0.6	16.7 ± 2.4

* Mean ± standard error.

their findings, Wollman and Scow (16) concluded that TSH affects the thyroidal iodide pump by means other than enhancement of the affinity of the transport carrier for its substrate, since it does not alter the K_M.[2] Comparison of the behavior of gastric and thyroidal iodide transport systems under conditions of loading with their substrate (Table 2) shows that the J/S declines less steeply with increasing serum iodide levels than does the T/S. A log-log plot of the data demonstrates that the K_M for the gastric iodide transport system is around 3 μg. of serum I^{127}/0.1 ml. vs. 1 μg./0.1 ml. for the thyroidal pump. This indicates that the gastric pump actually has a *lower* affinity for its substrate than that of the thyroid, which accounts for the fact that J/S and T/S are equal at physiologic serum I^{127} levels even though the capacity of the gastric juice for iodide is higher.

Earlier studies (18, 19) on dogs have also shown that the gastric iodide pump can be saturated with iodide. However, in the dog J/S values are depressed to 1 or below when the serum I^{127} reaches 3 mM/l. (=38.1 μg./0.1 ml.) (19), whereas in the rat the J/S is still significantly above 1 when the serum I^{127} levels are four times as high.

It is not known whether the stomach of the rat concentrates perchlorate. The thyroid of several species has been found not to concentrate inhibitors of iodide transport such as thiocyanate or perchlorate (17, 20, 21). Freinkel and Ingbar (21) have therefore postulated that two carriers make up the thyroidal iodide pump: the first accepts competing anions as well as iodide, the second is specific for iodide. A similar situation may exist in the stomach wall, or gastric iodide transport may be accomplished by a single carrier. In any event, the T/S is more readily depressed than the J/S whether graded doses of I^{127} or perchlorate are given. This appears to indicate that the reason for the observed kinetic difference between the gastric and the thyroidal iodide pump resides in sites to which both iodide and perchlorate attach. It is also evident (compare Tables 2 and 3) that perchlorate is about 10 times as effective as iodide in depressing the J/S. The same is true for the T/S, which confirms earlier findings (2). Apparently perchlorate has a greater affinity for or easier access to the sites on which it acts when it inhibits thyroidal or gastric iodide transport.

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[2] The K_M, which is inversely proportionate to the affinity of the pump for its substrate, is the serum iodide concentration which results in a T/S or J/S that is one-half of the difference between the value obtained without loading with iodide and the value observed when the pump is completely blocked.

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OBSERVATIONS CONCERNING THE HETEROGENEITY OF OVINE THYROGLOBULIN[1]

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ABSTRACT

Preparations of thyroglobulin have been made by salting-out techniques from the thyroid gland of a single sheep, labeled with I^{131} *in vivo*, from multiple thyroid glands labeled *in vitro*, and from multiple unlabeled glands. Despite electrophoretic and ultracentrifugal homogeneity, such preparations displayed evidence of heterogeneity, as assessed by their behavior during ion-exchange chromatography, ultraviolet absorption spectra, rate of turnover of I^{131}, and amino acid content.

THE thyroid gland is an organ specialized to perform two principal biosynthetic functions. Great attention has been directed toward the first, *i.e.*, the formation of iodinated amino acids capable of exerting characteristic metabolic effects at distant sites within the organism. However, the second major biosynthetic function of the gland, the synthesis of a specific protein framework within which these iodinations may occur, has been largely overlooked. Yet, there is evidence that several of the characteristics of this protein may contribute greatly to normal hormonal economy. Although *in vivo* iodination of tyrosyl groups within proteins may occur in organs other than the thyroid gland, these extrathyroidal iodoproteins do not contain hormonally active iodinated amino acids (1–3) The demonstration that the hormonally active iodothyronines (as well as their iodotyrosyl precursors) are situated as end groups within the thyroglobulin molecule (4) suggests that the configurational characteristics of the thyroglobulin molecule may somehow facilitate their formation. In addition, the large molecular size of thyroglobulin seems certain to make the task of the thyroid gland in maintaining a large store of hormone more

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[2] Much of this work was done during tenure as a Visiting Worker, National Institute for Medical Research, London, England. Present address, Thorndike Memorial Laboratory, Boston City Hospital, Boston, Massachusetts.

easily accomplished, and further, the capacity of thyroglobulin to hold thyroxine in a loose surface bond may afford some degree of regulation over the rate of hormonal release (5).

For these reasons, it seemed possible that a study of the synthesis of thyroglobulin might ultimately contribute to a better integrated understanding of the function of the normal and pathological thyroid gland. Before undertaking such a study, however, it seemed advisable to scrutinize more carefully the properties of the specific protein moiety in question.

Several unique characteristics of thyroglobulin, including its high concentration in a readily available tissue, its apparent relative ease of purification, and its large molecular size, have caused it to be employed as a model for the study of protein behavior in a number of systems, physicochemical as well as immunological (6–9). As a result, many of the physical constants and certain aspects of the chemical composition of thyroglobulin have been evaluated, and these in turn have served as criteria for assessing a number of methods for its isolation. Relatively recently, it has been shown that salting out techniques are capable of providing, from glandular extracts, preparations of apparently undenatured protein which have the physical and chemical characteristics of thyroglobulin (10). Although such preparations display electrophoretic and ultra-centrifugal homogeneity, they seem regularly to consist of several distinct moieties, as judged by solubility in salt solutions of high ionic strength (10). Attempts to assess the homogeneity of such preparations by other criteria, and to isolate the heterogeneous components, if possible, constitute the substance of the present report.

METHODS

Preparation of Thyroglobulin: Several batches of thyroglobulin were prepared from extracts of sheep thyroid glands by the method of Derrien, Michel, and Roche, employing ammonium sulfate as precipitating agent (10). Final preparations were extensively dialyzed against phosphate buffer, 0.01 *M*, pH 6.8, and were divided into aliquots which were frozen for use in individual experiments. This method of storing the protein was selected in view of the earlier demonstration that thyroglobulin loses its solubility after lyophilization (6).

The following batches of thyroglobulin were prepared:

Batches 1, 2, and 3: Thyroglobulin was obtained from pooled glands of several freshly killed sheep.

Batches 4 and 5: Twelve thyroid glands were obtained at the abattoir from freshly killed sheep. These were transported to the laboratory under refrigeration. Two glands were quickly sliced with a Stadie-Riggs microtome. Slices were distributed among a number of incubation flasks containing Krebs-Ringer-phosphate medium and a total of 500 μc. of inorganic I^{131}. After several hours of incubation at 38° C in an atmosphere of 100% oxygen, slices were removed, blotted, and pooled with slices which had been prepared from the remaining ten thyroid glands. The saline extracts of these glands was used to prepare Batches 4 and 5.

Batch 6: A single sheep was injected subcutaneously with 2 mc. of inorganic I^{131}. Twenty-four hours later the sheep was killed with a captive-bolt killer. The thyroid was quickly removed, freed of fat and fascia, and sectioned with Stadie-Riggs microtome.

Ion Exchange Chromatography: Ion exchange chromatography of preparations of thyroglobulin was performed, employing DEAE-cellulose, prepared according to techniques described by Peterson and Sober (11). Columns 1.5 cm. in diameter and 15 cm. long were most commonly employed. DEAE-cellulose was equilibrated with buffer (phosphate 0.01 *M*, pH 6.8) by means of a batch technique, following which the column was poured. Columns were packed and flow maintained under pressures of approximately 4 to 10 cm. of mercury, using either compressed air or nitrogen. After packing, the column was washed with another 50 ml. of buffer. The solution of protein, after dialysis against buffer of the same pH and molarity, was applied to the column, usually in a volume of 5 to 10 ml. An additional 50 to 100 ml. of starting buffer was than allowed to wash the column, and the total column effluent was collected during this period. In no case did significant quantities of radioactivity or protein pass through the column during the washing period.

Several experiments were performed in order to define optimum conditions for gradient elution. Ultimately, a gradient to buffer containing phosphate 0.05 *M*, NaCl 0.20 *M*, pH 5.7 was chosen. This buffer was passed from a reservoir into a mixing chamber containing 250 ml. of the starting buffer continuously agitated by a magnetic stirrer. Buffer was then passed to the top of the column. Fractions were collected in an automatic (constant interval) fraction collector, the flow-rate being adjusted by regulating the air pressure applied to the buffer reservoir. Flow was adjusted to a rate of approximately 20 ml./hour, and one fraction was collected every 15 minutes. Under these conditions, a complete elution could readily be performed overnight. Because of small variations in rates of flow, the volume of the individual fractions varied slightly.

In three experiments, the recovery of protein and radioactivity from the column was assessed. This was performed by analysis of the area under the elution curves. In all instances, recovery of radioactivity and protein exceeded 90%.

Analysis of Radioiodinated Amino Acid Content of Eluted Fractions: Protein fractions were precipitated from their eluting buffer with ammonium sulfate, were redissolved in urea-ammonium chloride solution, pH 8.5, and were subjected to tryptic hydrolysis at 38° C for twenty-four hours. Whole hydrolysates were then chromatographed on Whatman No. 1 paper in both butanol-dioxane-ammonia and butanol-acetic acid-water systems, using inorganic iodide, mono- and diiodotyrosine, triiodothyronine, and thyroxine as carriers. Carrier substances were localized with palladium chloride, in the case of iodide, and with diazotized sulfanilic acid, in the case of the iodinated amino acids. Localization of radioiodinated amino acids was determined by radioautography and by scanning the chromatographic strips in a continuous-feed-strip scanner in circuit with a rectilinear recorder. Quantitative assessment of the radioactivity in individual peaks in the chromatogram was obtained by analysis of their area.

Immunologic Techniques: Antisera against sheep thyroglobulin were prepared in three rabbits as follows: Solutions of thyroglobulin were suspended in Freund's adjuvant (light liquid paraffin, lanolin, and ground, heat-killed tubercle bacilli) to a final concentration of 6.25 mg./ml. Rabbits were given two intramuscular injections of 1.0 ml. of antigen one week apart. Following a rest period of four weeks, rabbits were twice injected intravenously with 5 mg. of thyroglobulin as alum-precipitated protein. Antisera were harvested two weeks later.

Miscellaneous Techniques: Solutions of thyroglobulin (1.3 gm.%) were electrophoresed in the 2.0 cm. cell of the Perkin-Elmer apparatus following prolonged dialysis against 0.05 *M* phosphate buffer, pH 6.8. Filter paper electrophoresis was performed in Whatman No. 3 paper in veronal buffer, pH 8.6, ionic strength 0.05. Spectrophotometric measurements were performed in a Unicam spectrophotometer, employing 1.0 cm. quartz cells. Nitrogen analyses were performed by a modified micro-Kjeldahl method. Measurements of radioactivity were carried out under conditions of constant

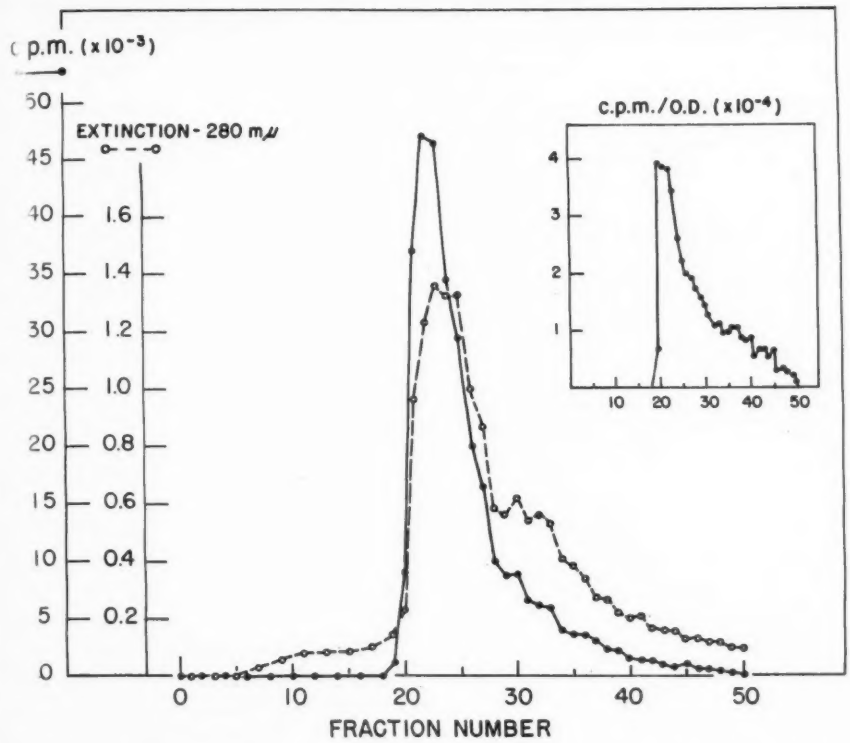

FIG. 1. Gradient elution of thyroglobulin from multiple sheep thyroid glands, labeled with I^{131} *in vitro*, from DEAE-cellulose resin. See text for chromatographic conditions.

geometry in a well-type scintillation counter. Sufficient counts were obtained to reduce the probable error of counting to less than 1%. Analyses for stable iodine were performed by the method of Barker, Humphrey, and Soley (12).[3] Proteins were analyzed for amino acid content by the technique of Moore and Stein (13), following hydrolysis in 6N HCl.

RESULTS

Batch 4 consisted of thyroglobulin extracted from twelve sheep thyroid glands, of which two had been allowed to accumulate I^{131} *in vitro* during an incubation period of several hours. Ion exchange chromatography of Batch 4 produced results depicted in Figure 1. Two principal findings were noted. First, the curve of protein elution was asymmetrical, displaying a rather sharp peak, followed by a distinct shoulder and tail. Second, fractions associated with the primary protein peak contained considerably more radioactivity than fractions in the shoulder and tail of the elution curve. Where-

[3] Performed by Bioscience Laboratories, 231 South Carmelina Avenue, Los Angeles 64, California.

as proteins in the shoulder and tail of the elution curve contained approximately 40% of the ultraviolet absorbing moieties recovered from the column, they contained only 20% of the radioactivity. Calculation of the ratio of radioactivity to extinction at 280 mμ. for successive fractions of the elution curve revealed that peak ratio was achieved in the first fraction which contained appreciable protein. The ratio remained virtually constant in the next two fractions and then declined progressively, varying approximately seven-fold between the peak and the latest-eluting fractions.

Consecutive fractions in the elution curve were mixed to form several pools (20–22, 23–25, 26–30, 31–36, 37–47) in which ultraviolet absorption

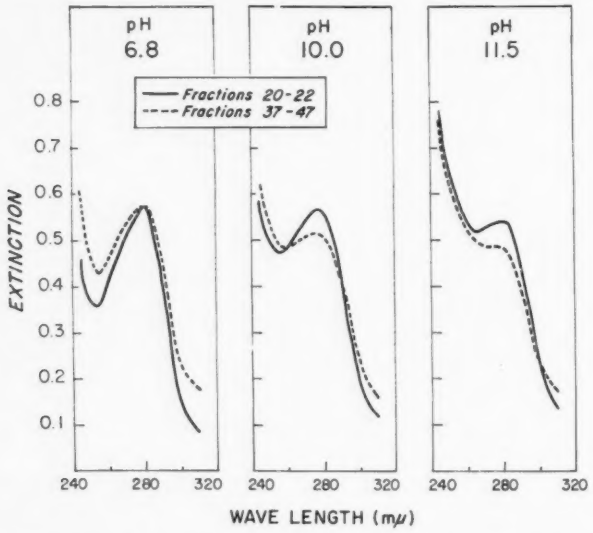

FIG. 2. Ultraviolet absorption spectra of pooled fractions of thyroglobulin from the elution curve shown in Figure 1.

spectra were determined. Results obtained in fractions 20–22 and 37–47 are illustrated in Figure 2. Maximum differences in absorption spectra were observed in pooled fractions from the extreme ends (20–22 and 37–47) of the elution curve (Fig. 2). The spectral curve shifted progressively from that displayed by fractions 20–22 toward that displayed by fractions 37–47 as pools from successive portions of the elution curve were examined. Early fractions displayed a distinctly lesser absorption in the 250–255 and in the 300–310 mμ. range than did fractions eluted late. Early fractions also displayed a distinctly sharper absorption peak at precisely 280 mμ. The spectrum of pooled fractions 37–47 revealed a broak peak which was almost flat between 278 and 282 mμ. Differences among the various pools diminished as the pH at which observations were made was increased. Maximum changes in absorption during change in pH occurred in the 245–255 mμ.

ranges, were most marked when the pH was increased from 10.0 to 11.5, and were of greatest magnitude in pooled fractions 20–22.

Batch 5, representing a second preparation of thyroglobulin from the same saline extract as that which yielded Batch 4, gave entirely identical results with those obtained in Batch 4, both as regards the shape of the elution curve for radioactivity and for protein and as regards spectral absorption curves.

Pools were made of fractions in the early and in the late portion of the elution curve of Batch 5. These were dialyzed against 0.01 *M* phosphate buffer, pH 6.8, and were subjected to ultracentrifugal analysis (Fig. 3).

FIG. 3. Ultracentrifugal analyses of pooled fractions of thyroglobulin from the elution curve shown in Figure 1.

Except for a small, rapidly-sedimenting moiety in the late-eluting fractions, both fractions appeared homogeneous. Calculated values for $S_{20,w}$ were 19.2 and 19.7, respectively, for the early and late-eluting fractions. No moieties displaying less rapid sedimentation were seen.

When subjected to chromatography on the DEAE-cellulose column, Batch 2 of unlabelled thyroglobulin displayed an asymmetrical elution curve similar to that observed with Batches 4 and 5 (Fig. 4). Fractions 18–20 and 35–41 were dialyzed as separate pools against 0.01 *M* phosphate buffer, pH 8.6, and following dialysis, their absorption at 280 mμ. was determined. Known volumes of the two pools were combined into a single pool which was then chromatographed on the exchange column. During elution, two peaks appeared, corresponding very closely to the position of the fractions taken from the original elution curve, when correction was made for variation in the volume of the individual fractions. Calculated values revealed very good agreement between the quantity of U-V absorbing material recovered in each of the two peaks and the quantities applied from the corresponding portions of the original elution curve.

Batch 6, prepared from a single sheep thyroid gland, labelled with I^{131} *in vivo*, was chromatographed on the DEAE-cellulose column under standard conditions. Results are illustrated in Figure 5. The elution pattern obtained was virtually identical with that seen in Batches 4 and 5. Both asymmetry of the protein elution curve and non-uniform labelling were seen.

Differences in absorption spectra between early- and late-eluting portions of the curve in Batch 6 were the same as those described for Batches 4 and

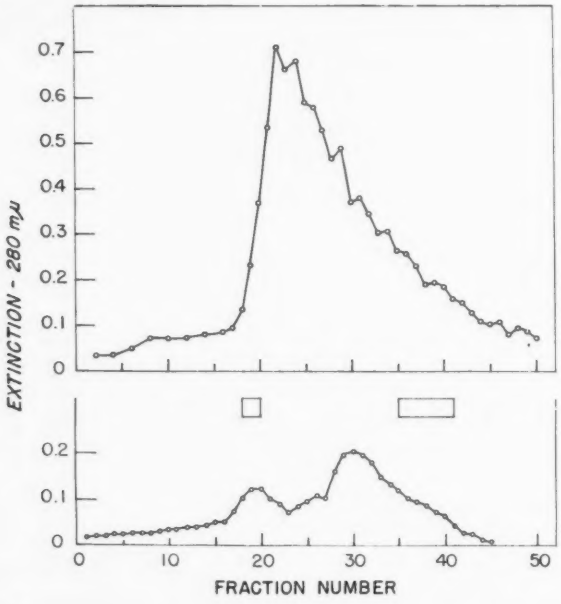

FIG. 4. Gradient elution of unlabeled thyroglobulin from multiple sheep thyroid glands. Fractions in the area indicated by the two rectangles were pooled and rechromatographed, with the results shown in the lower elution curve.

5, preparations which contained thyroglobulin from the glands of multiple animals.

Tryptic hydrolysis and paper chromatography of alternate fractions of the entire elution curve revealed that each fraction contained iodide, mono- and diiodotyrosine, thyroxine and triiodothyronine, as well as other unidentified components. Among the several fractions, the relative proportion of iodinated amino acids of each type did not differ significantly.

Stable iodine analyses were performed on Fractions 21 and 31 of the elution curve of Batch 6. Iodine/nitrogen ratios were 28.4 μg./mg. in Fraction 21 and 26.6 μg./mg. in Fraction 31. Specific activity of iodine in Fraction 21 was calculated to be four times as great as that in Fraction 31. Similar results were obtained when iodine and nitrogen analyses were ob-

ained in early- (28.7 μg./mg.) and late-eluting (31.2 μg./mg.) fractions of Batch 3.

Amino acid analysis of early- and late-eluting fractions from Batch 6 evealed small differences in the percentage composition of a number of mino acids (Table 1). Marked differences in two respects were observed. First, early eluting fractions contained considerably more valine, and econd, late-eluting fractions contained a component which was completely absent from early-eluting fractions. This component has been

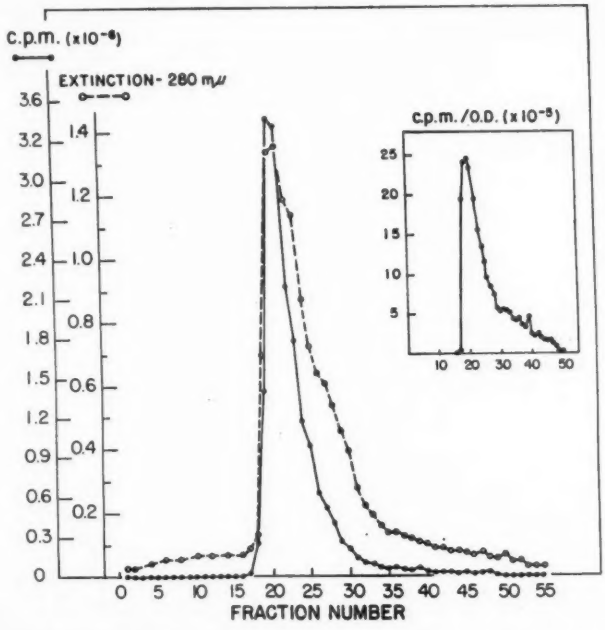

FIG. 5. Gradient elution of thyroglobulin from a single sheep thyroid gland, labeled with I^{131} *in vivo*, from DEAE-cellulose resin. Chromatographic conditions the same as those in Figure 1.

tentatively identified as a hexosamine. The content of arginine was not determined in early-eluting fractions, as tubes containing that portion of the amino acid elution pattern were lost.

Study of the immunologic complexity of the whole thyroglobulin-anti-thyroglobulin system was performed by the Ouchterlony agar plate-diffusion technique (14). In this system, two distinct bands were seen in the zone of interaction between antiserum and whole, unfractionated thyroglobulin (the original antigen). The same two bands were found in both early- and late-eluting fractions from Batches 3 and 6. A small band was also seen when antiserum was allowed to interact with sheep serum, but this band was not confluent with those obtained during the interaction with thyroglobulin.

TABLE 1. AMINO ACID CONTENT OF EARLY- AND LATE-ELUTING FRACTIONS OF OVINE THYROGLOBULIN

	I Fractions 18–20*	II Fractions 28–31	III Fractions 28–31
Aspartic	7.8	9.0	8.4
Threonine	4.8	5.7	5.3
Serine	7.6	8.2	7.6
Glutamic	15.1	16.6	15.5
Proline	7.3	7.5	7.0
Glycine	5.0	5.3	4.9
Alanine	6.5	6.0	5.5
Cystine	1.1	0.9	0.8
Valine	9.5	5.7	5.3
Methionine	1.2	1.2	1.1
Isoleucine	2.6	2.6	2.4
Leucine	11.7	10.8	10.1
Hexosamine	0.0	1.9	1.8
Tyrosine	3.3	3.2	3.0
Phenylalanine	7.8	7.0	6.5
Lysine	4.2	3.5	3.2
Ammonia	2.2	2.5	2.3
Histidine	2.4	2.4	2.2
Arginine	—	—	7.1

* Tubes of Moore and Stein effluent containing arginine lost. Therefore, for purposes of comparison to each other, values in fractions 18–20 and 28–31 (Columns I and II) calculated on basis of content, exclusive of arginine. For purposes of comparison with other values in the literature, Column III presents values for fractions 28–31, including arginine.

DISCUSSION

The initial observation of inhomogeneity and non-uniform labeling in the thyroglobulin extracted from a pool containing the glands of several animals suggested that, despite its electrophoretic homogeneity, this preparation might be contaminated with another protein which was labeled little, if at all. This possibility seemed to be excluded by the ultracentrifugal experiments. These demonstrated that pools of both highly-labeled and slightly-labeled fractions had similar sedimentation constants, both being in the characteristic range for whole thyroglobulin (6, 8, 9). Even if it had contained no radioiodine at all, the small rapidly-sedimenting moiety found in the slightly-labeled fractions could not, by virtue of its low concentration, have accounted for the low I^{131}/N ratio of these fractions. Ultracentrifugal observations also served to exclude partial denaturation or hydrolysis of the protein as the cause of the apparent inhomogeneity, since neither pool displayed the characteristic alterations in sedimentation behavior which occur under these conditions (15, 16).

Further possible explanations were assessed in experiments performed with thyroglobulin prepared from a single sheep gland labeled *in vivo*. The striking similarity between the elution curves in this preparation and in thyroglobulin prepared from multiple glands served to exclude animal to animal variation as a source of inhomogeneity.

Another possibility was assessed by means of the material labeled *in vivo*.

It has been reported that during *in vitro* incubation of thyroid slices in media containing inorganic I^{131}, the principal radioiodinated products are iodotyrosines, while iodothyronines are labeled little if at all (17). Possibly, preparations of thyroglobulin might contain a mixture of proteins, similar in most respects, but varying markedly in their relative content of iodotyrosines and iodothyronines. In tissue labeled *in vitro*, proteins rich in iodotyrosines would therefore be much more highly labeled than proteins containing predominantly iodothyronines. Furthermore, the pK's of the iodotyrosines are higher than those of the iodothyronines and proteins rich in the former might be expected to elute from the column at a higher pH, *i.e.*, earlier in the elution curve. These considerations might explain the findings obtained during chromatography of thyroglobulin labeled *in vitro*.

During *in vivo* labeling, radioiodinated thyroxine and triiodothyronine were formed. It was possible to demonstrate by paper chromatography that the fractions obtained throughout the elution curve all contained iodotyrosines and iodothyronines in approximately the same proportions. This did not prove conclusively, however, that the stable iodinated amino acids in all fractions were quantitatively and qualitatively the same, since the radioactive components found in late-eluting fractions may merely have been portions of the early-eluting fractions which trailed off the column. Nevertheless, I^{127}/N ratios in the two extremes of the elution curve were virtually identical, indicating that, whatever their chemical nature, iodinated moieties in the late-eluting fractions were turning over very slowly.

It also seemed possible that the curve of protein elution might represent the behavior of a single species of protein, and that the asymmetry observed was merely a function of the chromatographic system. This possibility was rendered unlikely by experiments performed with unlabeled thyroglobulin, Batch 3. Fractions from the two extremes of the elution curve were pooled and rechromatographed. During rechromatography, two peaks were obtained. Had asymmetry of the elution pattern resulted merely from trailing of a homogeneous protein, a single, asymmetrical peak, similar to the first, would have been expected during rechromatography. The appearance of two peaks, however, suggested the presence of two distinct groups of components, chromatographing independently.

This experiment, employing unlabeled thyroglobulin, further indicated that the asymmetry observed in previous experiments could not be explained by radiation damage to the protein.

As a final criterion of heterogeneity, amino acid analyses were performed on fractions from the extreme portions of the elution curve of thyroglobulin from a single gland (Batch 6). Since hydrolysis of protein was carried out in 6N HCl, analyses for tryptophane could not be obtained. Small differences in the percentage composition of a number of other amino acids were

seen, and striking differences were found in the content of valine and of another component, probably a hexosamine. The latter component was found only in the late-eluting fractions. In general, values for the various amino acids in both groups of proteins agreed well with those previously reported for the thyroglobulins of other species, studied by a number of different techniques (9, 18). One possible discrepancy between the present values and those reported earlier was found in the case of arginine. This has been reported to be the preponderant amino acid in thyroglobulin, values as high as 12.7% having been noted (9). In the present study, arginine constituted but 7% by weight of the proteins in the late-eluting fractions. Unfortunately, loss of the appropriate samples prevented analysis for arginine in the earlier fractions. It is apparent, however, that the arginine content of these fractions would necessarily have been very high in order for the average arginine content of the entire batch to have been 12%. Alternatively, the low arginine content of the late-eluting fractions might be explained by the relative resistance of some of the arginine bonds in thyroglobulin to acid hydrolysis (19). A further difference between the present and previously noted values was seen in the content of valine. Present values of from 5.7 to 9.5%, obtained in the sheep, are far higher than those reported for the porcine protein (9).

Considered together, the present observations thus indicate that even within the thyroid gland of a single animal, thyroglobulin is not entirely homogeneous, but rather may be a complex mixture of closely related proteins.[4] Preliminary studies have revealed similar complexity in thyroglobulin extracted from human material (20). The precise relationship of the present findings to the earlier demonstration that thyroglobulin appears to be composed of three moieties, as judged by solubility characteristics, remains unclear. Although many of the observations herein presented were made in fractions expected to display maximum differences, it seems likely that between these extremes there lies a spectrum of closely related proteins. It is noteworthy that the present techniques, although effective in separating these moieties at least partially, did not resolve the apparent immunological complexity herein demonstrated for sheep thyroglobulin, and previously noted in human material (21).

The physiological significance of these findings is obscure. However, one possible explanation for heterogeneity of thyroglobulin arises from the observation that within a single thyroid gland, individual follicles may show differing degrees of activity (22, 23). It seems possible, therefore, that the different thyroglobulins noted in the present study may be the products of follicles of varying function. Extension of studies to include the thyro-

[4] Attempts to correlate the chromatographic behavior of the various fractions with their amino acid and carbohydrate contents, and with their spectral absorption curves, are beyond the scope of the present communication, and will be presented elsewhere.

globulin of normal and abnormal human or animal glands might clarify his relationship. In addition, the function, if any, of protein-bound carbohydrate, including hexosamine, in which the thyroid gland is relatively rich (18, 23–25), seems to merit attention, especially since hexosamine was found only in fractions of thyroglobulin whose iodine was turning over slowly.

Finally, it is of interest that differences in rates of iodination are accompanied by variations in the chemical composition or configuration of thyroglobulin. Recent studies suggest that the synthesis of iodinated amino acids *in vivo* cannot be divorced from the synthesis of thyroidal protein, and that the two processes are either related, or at least occur *pari passu* (20). Relevant to these observations, is the demonstration of abnormal iodoproteins in the plasma of some patients with thyroidal carcinoma (26), congenital goiter (27), or adult non-toxic goiter (28). Abnormal thyroglobulins have also been found in carcinomatous thyroid tissue (29) In these abnormal proteins, hormonally active iodinated amino acids are not found. Thus, the relation between abnormal protein synthesis and disordered hormonal biogenesis would seem to merit further investigation. Such studies are now in progress.

Acknowledgments

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THE EFFECT OF VARIOUS THYROXINE ANALOGUES ON SUPPRESSION OF I^{131} UPTAKE BY THE RAT THYROID[1]

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ABSTRACT

Forty-five different thyroxine analogues have been compared to *l*-thyroxine for their ability to decrease the thyroid collection of I^{131} in the rat. On a weight basis only 3,5,3′-triiodo-*l*-thyronine was found more effective than *l*-thyroxine. Compounds containing iodine were all capable of producing some inhibition providing the dosage was adequate.

THE dose of thyroid hormone, within limits, can be directly related to thyroidal uptake of I^{131} in experimental animals. This method has been used in the present report to assay the relative effectiveness of thyroxine analogues in blocking uptake of radioiodine in rats.

METHODS AND MATERIALS

Approximately 2,500 male rats of the Sprague-Dawley strain (Charles-River Breeding Laboratories, Boston, Massachusetts) ranging in weight between 75–125 gm. were used in this study. They were selected so that the average body weight of each group was essentially identical within each separate experiment. At the start of each study animals were placed on a low iodine diet (1) and given 0.1% thiouracil in drinking water. After 5 days of this treatment the water containing thiouracil was replaced with ordinary tap water which was continued to the termination of the experiment. Twenty-four hours after the thiouracil was stopped, daily subcutaneous injections of the test analogues were started. These were administered for 5 days and 24 hours after the last injection all animals were given a single intraperitoneal injection of 0.1 μc. of I^{131}. These animals along with a control group of a similar number of uninjected rats and a group maintained on regular laboratory diet were autopsied 24 hours after the administration of I^{131}. At autopsy the thyroids were removed, dissected free of extraneous tissue, weighed on a Roller-Smith balance and placed in 5 cc. vials containing a standard volume of Bouin's solution. Radioactivity was determined using a well-type scintillation counter and the thyroid uptake of I^{131} calculated using an appropriate standard.

In any given experiment 3 doses of each analogue (dissolved as previously described (2) and injected in a volume of 1 cc.) were assayed with 5–10 animals per group. Calculations for relative potency compared with *l*-thyroxine were made as follows. Actual data obtained from an assay were plotted on semi-log paper and the dose of the analogue

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TABLE 1. RELATIVE INHIBITORY ACTIVITY (R.A.) OF VARIOUS ANALOGUES IN BLOCKING UPTAKE OF I^{131} BY THE RAT THYROID

Compound	RA
Thyronine series	
l-thyroxine	100
d-thyroxine	50
dl-thyroxine	40
3,5,3′,5′-tetrachloro-dl-thyronine	<1.5
3,5,3′-triiodo-l-thyronine	300
3,3′,5′-triiodo-dl-thyronine	75
3,5-diiodo-l-thyronine	37
3,3′-diiodo-dl-thyronine	100
3-iodo-dl-thyronine	50
1-nitro-5-(3,5-dinitrothyromethylene) hydantoin, methyl ether	<3
Butyric series	
3,5,3′,5′-tetraiodothyrobutyric acid	30
3,5,3′-triiodothyrobutyric acid	75
3,5-diiodothyrobutyric acid	60
Acrylic series	
3,5,3′,5′-tetraiodothyroacrylic acid	50
Propionic series	
3,5,3′,5′-tetraiodothyropropionic acid	60
3,5,3′-triiodothyropropionic acid	60
3,3′,5′-triiodothyropropionic acid	50
3,5-diiodothyropropionic acid	16
3,5-diiodothyromethylmalonic acid, methyl ether, diethyl ester	20
3,3′-diiodothyropropionic acid	25
3-iodothyropropionic acid, methyl ether	17
3-aminothyropropionic acid	Inactive
3-aminothyropropionic acid, methyl ether	<0.3
3-aminothyropropionic acid, methyl ether, ethyl ester	Inactive
3,5-diaminothyromethylmalonic acid, methyl ether, diethyl ester	Inactive
3-nitrothyroacrylic acid, methyl ether	<1.5
3-nitrothyroacrylic acid, methyl ether, ethyl ester	Inactive
3,5-dinitrothyromethylenemalonic acid, methyl ether, diethyl ester	<1.5
Acetic series	
3,5,3′,5′-tetraiodothyroacetic acid	75
3,5,3′-triiodothyroacetic acid	75
3,3′,5′-triiodothyroacetic acid	15
3,5,3′-triiodothyroacetic acid, methyl ether	37
3,5-diiodothyroacetic acid	18
3,5-diiodothyroacetic acid, methyl ether	50
3,3′-diiodothyroacetic acid	25
3-iodothyroacetic acid	33
3,5-diaminothyroacetic acid, methyl ether	7.5
3,5-dinitrothyroacetic acid, methyl ether	Inactive
3,5,3′-trinitrothyroacetic acid, methyl ether, ethyl ester	Inactive
Formic series	
3,5,3′,5′-tetraiodothyroformic acid	10
3,5,3′-triiodothyroformic acid	15
3,5-diiodothyroformic acid, methyl ether, methyl ester	15
3,5,3′-triaminothyroformic acid, methyl ether, methyl ester	Inactive
3,5-diaminothyroformic acid, methyl ether, methyl ester	Inactive
3,5-dinitrothyroformic acid, methyl ether	Inactive
3,5-dinitrothyroformic acid, methyl ether, methyl ester	Inactive

necessary to produce a control (untreated animals on regular diet) uptake of radioiodine read off from the graph. The dose of *l*-thyroxine necessary to produce this effect was consistently between 10 and 15 μg. This figure was arbitrarily assigned a value of 100, and calculations were made for the analogues to give a relative potency in terms of *l*-thyroxine. These values are shown in Table 1. A compound showing no ability to lower I^{131} uptake even in doses of 500 μg. daily is reported as inactive.

RESULTS AND DISCUSSION

The data in Table 1 were obtained using 46 different compounds. Most of these compounds were also tested at the same time for their ability to produce metamorphosis of the tadpole as has been reported (2). Similarly, the determination of the antigoitrogenic and calorigenic activities of the analogues have been determined in rats. These latter studies are reported in the present issue of *Endocrinology*. The reader may find the structural formulae of the compounds in Table 1 by referring to the paper of Stasilli *et al.*

It is apparent from Table 1 that under the conditions used only 3,5,3′-triiodo-*l*-thyronine is more effective on a weight basis than *l*-thyroxine in blocking the uptake of I^{131} by the rat thyroid. Whether other compounds would prove to be more effective at times other than 24 hours after I^{131} was not determined. It is of interest that only one analogue showed the same relative inhibitory activity as *l*-thyroxine.

It seems rather unlikely that the number of atoms of iodine in the molecule is the chief factor involved in blocking thyroidal I^{131} collection. For example, *d*-thyroxine appears to be only one-half as effective as the *l*-isomer while *d*-*l*-thyroxine is no more active than the *d*-form. In the triiodinated compounds, as was found in tadpole metamorphosis (2), in general the 3,5,3′-analogues appear to be somewhat more effective than the 3,3′,5′ compounds. All analogues which contained iodine manifested various degrees of inhibitory effect on the uptake of I^{131} when given in adequate doses. On the other hand few non-halogen containing compounds showed evidence of inhibitory activity.

Other studies (2, 3) have pointed out the difficulties encountered in studies using thyroxine analogues. The major problems in obtaining valid data are the purity and stability of the compounds. The latter problem has been considered (3) in some detail and was realized when the present studies were done. However, as has been pointed out (3) degradation of some of these compounds does occur. How much and in what way the present results may have been altered by this degradation is at present not certain.

Acknowledgments

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THE *IN VITRO* METABOLISM OF THYROXINE, TRIIODOTHYRONINE AND THEIR ACETIC AND PROPIONIC ACID ANALOGUES[1]

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ABSTRACT

The metabolism of thyroxine, triiodothyronine and their acetic and propionic acid analogues has been studied by placing these I^{131} labelled substrates in buffered medium with or without added tissue. Spleen, kidney and liver slices, homogenates and fractions of homogenates were used. Samples of medium and/or homogenized slices, usually after three hours' incubation at 37° C, were chromatographed and quantitated by strip counting. The only product of deiodination detected was iodide. When thyroxine endogenously labelled with I^{131} was used, no 3, 5 diiodothyronine or other partially deiodinated thyronines or derivatives were formed, although activity was low and small amounts of other products could not be ruled out. Deiodination was inhibited by a nitrogen atmosphere, boiling, heavy metals, cyanide, 2 methyl 1, 4 naphthoquinone, a chelating agent (Versene), and estrogenic substances. No antithyroid substances, including iodide and propylthiouracil, had any effect; iodinated tyrosines and organic iodine compounds had no effect. All tetra and tri iodinated thyronines inhibited the deiodination of other tetra and tri iodinated thyronines, often commensurate with the degree of their own deiodination. Only one iodinated compound, other than these, was an effective inhibitor: N-butyl para hydroxy diiodobenzoate.

IN VIVO and *in vitro* studies of thyroxine and triiodothyronine have indicated several pathways of metabolism involving deiodination, decarboxylation and deamination. Gross and Leblond (1) gave thyroxine labelled with I^{131} to thyroidectomized mice and found radiotriiodothyronine (unknown 1) in the organs. The conversion of thyroxine to triiodothyronine by kidney slices has been demonstrated (2), although the chroma ographic differentiation of triiodothyronine from the acetic or propionic acid analogue of thyroxine has been questioned (3). Deiodination of radiothyroxine and radiotriiodothyronine by rat skeletal muscle homogenates (4) resulted in the formation of radioiodide and small amounts of other unidentified products; whereas, rat brain homogenates (3) formed significant amounts of the acetic and/or propionic acid analogues in addition to iodide, and, occasionally, small amounts of triiodothyronine. Rat kidney homogenate and mitochondria incubated with radiothyroxine did not result in the formation of triiodothyronine or iodide but rather in the acetic acid analogue (5). Similar studies with radiotriiodothyronine indicated that the acetic acid analogue was the product formed (5). Roche (6)

[1] Presented at the 1958 Meeting of the American Goiter Association.

et al. have demonstrated that the kidneys of thyroidectomized rats treated with radiotriiodothyronine contain radiotriiodothyroacetic acid and (7, 8) that the pyruvic acid analogues are found in the urine and bile after the injection of radiothyroxine and radiotriiodothyronine. Taurog *et al.* (9) have indicated that conjugation of thyroxine with glucuronic acid occurs in the presence of liver slices.

Tissue deiodinating systems (2, 4) were inhibited by boiling. Anaerobic conditions (3, 4) did not affect the results but cyanide (2, 3), iodide, (2) and mercuric chloride (3) were effective inhibitors.

The present study on the metabolism by tissue preparations of thyroxine and triiodothyronine attempts to clarify the products formed, the effect of various substances added to the medium with particular emphasis on compounds known to affect the thyroid, enzyme inhibitors and other iodinated compounds, and the specificity of the enzyme system responsible for deiodination.

METHOD

Livers, spleens, muscle and kidneys were obtained from normal Sprague-Dawley male rats (200–250 gm.) killed by ether. Slices were cut freehand (0.5–1.5 mm.) and placed (200–300 mg.) in 2 ml. of modified Krebs-Ringer-phosphate solution ($CaCl_2$ $8\times10^{-4}M$) at pH 7.4 to which 2–3 μc. of I^{131} labelled (as obtained from Abbott Labs) *l*-thyroxine, 1–3,5,3′ triiodothyronine, tetraiodothyroacetic or propionic acid, 1–3,5,3′ triiodothyroacetic or propionic acid had been added. The tubes were incubated at 37° C with constant shaking for 3 hours. Substrate concentrations of $2.6\times10^{-5}M$ to $5.6\times10^{-2}M$ were obtained by adding the appropriate amount of carrier. Homogenates were similarly treated. Fractionation of tissue homogenized in $0.25M$ sucrose solution was accomplished by differential centrifugation (modified Hageboom). The following fractions, in a concentration of 400 mg. of tissue per 4 ml. were then treated as above: whole homogenate, nuclei and debris, mitochondria, microsomes, supernatant and a combination of mitochrondria, microsomes and supernatant. After incubation (slices were homogenized) an aliquot (20λ) was placed on paper and chromatographed in t-amyl alcohol saturated with $2N$ NH_4OH (descending) and/or n-butanol-acetic acid-water (78–10–12). In several experiments the five solvent systems noted below were used. Known carrier spots[2] were identified with ninhydrin or ceric sulfate-arsenious acid. The strips were exposed to x-ray film and counted on a strip counter (Nuclear-Chicago). Planimeter measurements were made for quantitation.

Thyroxine labelled endogenously with radioiodine was obtained by feeding rats propylthiouracil (0.3%) for 4 days and discontinuing it 18 hours prior to injecting 100 μc. of radioiodine. Twenty-four hours later the rats were sacrificed, thyroid glands removed, homogenized, hydrolyzed by pancreatin for 24 hours in K-R-P at pH 7.4 and extracted with butanol saturated with acidified water (pH 3.5). The extract was then evaporated, redissolved in a small amount of butanol and chromatographed in t-amyl alcohol saturated with $2N$ NH_4OH. The radio-thyroxine spot was eluted with butanol and acidified water, evaporated to dryness and redissolved in 50% propylene glycol. The iodine content of this material was $10^{-7}M$ as determined by a modified Barker technique. An aliquot of this endogenously labelled radiothyroxine was added to tubes with rat liver slices. At 6 hours a sample of the medium was placed on paper, the tissue was homoge-

[2] Thyroxine, 3,5,3′ triiodothyronine, and the propionic and acetic acid analogues were kindly supplied by Dr. Michael Barry of Warner-Chilcott Laboratories.

nized and placed on paper directly or extracted with butanol and acidified water. The extract was evaporated to dryness and redissolved in 0.5 cc. butanol and acidified water. Five solvent systems were used for chromatography: t-amyl alcohol-ammonium hydroxide, butanol-dioxane-ammonium hydroxide, collidine-water in an atmosphere of ammonia, butanol-acetic acid-water and butanol-ethanol-ammonium hydroxide. In one experiment the rats were injected with radioiodine without prior treatment with propylthiouracil. After incubation with pancreatin, the medium was directly chromatographed without extraction and the areas of thyroxine and triiodothyronine were cut out and placed directly in the control or liver tubes. At 4 hours the medium was placed on paper.

RESULTS

Deiodination of thyroxine and triiodothyronine and the products formed

The major portion of deiodination occurred within three hours and only minor changes occurred thereafter. Temperatures of 5° C completely inhibited and 50° C and 70° C reduced the amount of deiodination; 37° C

TABLE 1. DEIODINATION OF L-THYROXINE AND L-3,5,3′-TRIIODOTHYRONINE BY RAT LIVER, KIDNEY, AND SPLEEN SLICES (250 MG./3 CC.) INCUBATED FOR 3 HOURS

	L-thyroxine 7.6×10^{-7}M*			L-3,5,3′-triiodothyronine 2×10^{-8}M		
	% total distributed as			% total distributed as		
	T4	I^-	O†	T3	I^-	O
Control	80.6	9.2	10.2	95.5	3.6	0.9
Liver	35.4	54.6	10.0	65.7	27.7	6.6
Spleen	64.6	28.9	6.5	86.6	10.5	2.9
Kidney (Cortex)	44.3	50.0	5.7	64.0	30.7	5.3

* Note that concentration of T4 is 35×T3.

† Represents radioactivity staying at the origin in t-amyl alcohol saturated with 2N NH_4OH. In occasional experiments there were traces of unknowns and material at the front.

seemed to be optimal. Table 1 illustrates the degree of deiodination of thyroxine and triiodothyronine when incubated with liver, spleen, or kidney slices. Liver and kidney (and muscle) were active whereas spleen was only slightly active. At three hours there was more deiodination of thyroxine than triiodothyronine (Table 1), indicating that thyroxine was more rapidly deiodinated.

Iodide, released from the deiodination of thyroxine and triiodothyronine, was present in the medium. Small amounts of material travelling at the front were not considered significant. As much as 10% (and, on one occasion, 20%) of the total radioactivity remained at the origin on chromatography. Although small amounts stayed at the origin from tubes without tissue, the amount at the origin increased markedly in the presence of tissue. This was presumably iodinated protein or thyroxine and protein complexed so as to be insoluble in t-amyl alcohol, though it was not hydrolyzed and rechromatographed. Unidentified compounds occurred sporadically, the most frequent one having an R_f, in t-amyl-ammonium hydroxide, between those of iodide and thyroxine. This unidentified material was present

in the control tubes and diminished markedly after incubation with liver slices. When butanol-ethanol-ammonium hydroxide was used as a solvent, a radioactive spot just above the origin often appeared after incubation. Since this spot also appeared when radioactive iodine alone was chromatographed, it was felt to be an oxidized derivative of iodide. In some instances, chromatography of thyroxine labelled with radioiodine (Abbott) and incubated in control tubes for three hours without tissue would result in six distinct spots in t-amyl alcohol-2N NH_4OH while only two distinct spots (iodide and thyroxine) were noted in butanol-ethanol-2N NH_4OH. These spots of unknown compounds were all diminished in amount when the thyroxine was incubated with liver slices. A question arises as to whether these various spots represent compounds originally present or factitiously produced by the action of t-amyl alcohol. It is of interest that

TABLE 2. ACTIVITY OF LIVER FRACTIONS IN DEIODINATION OF L-THYROXINE

Tissue	L-thyroxine 2.6×10^{-6}M		
	% of total distributed as		
	T4	I^-	O
Slices	45.8	34.2	20.0
Homogenate	41.0	47.3	11.7
Nuclei and debris	64.8	24.1	11.1
Mitochondria	39.0	42.7	18.3
Microsomes	74.9	20.8	4.3
Supernatant fluid	58.3	29.7	12.0
Mitochondria, microsomes and supernatant	32.2	56.0	11.8
Control	75.2	16.2	8.6

carrier thyroxine is more difficult to demonstrate by ninhydrin stain after chromatography with t-amyl alcohol solvent than with butanol-ethanol-ammonium hydroxide.

Localization of deiodination and the effects of anaerobiosis and boiling

Slices, homogenate, mitochondria, and the combination of mitochondria, microsomes and supernant were all active in the deiodination of thyroxine (Table 2). Nuclei and debris, microsomes and supernatant fluid were much less active indicating that the mitochondria was the most active of the cell fractions. Anaerobic conditions inhibited the deiodination of thyroxine by slices and mitochondria and of triiodothyronine by slices. Boiling ten minutes markedly reduced the deiodination of thyroxine by slices and mitochondria and of triiodothyronine by slices. Thirty minutes boiling of the homogenate did not affect the deiodination of thyroxine or triiodothyronine, although sixty minutes of boiling inhibited to a moderate extent.

Effect of substrate concentration

Increasing the concentration of *l*-thyroxine and *l*-triiodothyronine above physiological levels markedly reduced the per cent of radioactive material

deiodinated by slices and homogenates. At 10^{-5} *M* the per cent deiodinated of both is so low as to be unmeasurable. At 10^{-7} *M* thyroxine is 80–90% deiodinated. Triiodothyronine at 10^{-8} *M* is only 50% deiodinated. There is, then a marked difference between the quantity of thyroxine and triiodothyronine which can be deiodinated by liver slices or homogenates during an equal period of time.

Inhibition by various compounds of deiodination

The deiodination of thyroxine and triiodothyronine by liver slices and homogenates is markedly inhibited by mercuric chloride and n-butyl parahydroxy, 3,5-diiodobenzoate (BHDB) (Table 3). Deiodination by mitochondria is not, however, affected by BHDB (Table 3). Less marked, but definite, was the inhibition by other heavy metals (cupric chloride, cobaltous sulfate), some phenols (resorcinol, estradiol), a quinone (2-methyl 1,4-naphthoquinone), a chelating agent (ethylene diamine tetra acetic acid), and an oxidative enzyme inhibitor (sodium cyanide, not charted). The results with hydroquinone and silver nitrate were difficult to interpret due to their effect on the chromatographic characteristics of iodine compounds (10). The estrogenic substances, estrone and estradiol, were effective inhibitors whereas corticosterone, hydrocortisone, desoxycorticosterone, testosterone and progesterone were ineffective. Reserpine and thyronine had no effect. Sodium azide may have had a slight inhibitory effect but the following compounds had no effect: compounds which affect thyroid function (iodide, Tapazole, propylthiouracil, sulfanilamide, potassium thiocyanate), other iodinated compounds (iodide, monoiodotyrosine, diiodotyrosine, 3,5-diiodothyronine, Teridax,[3] Priodax,[4] Telepaque[5]), a specific sulfhydryl group inhibitor (parachloromercuribenzoate).

The deiodination of acetic and propionic acid analogues of thyroxine and triiodothyronine

The deiodination of the acetic acid analogues of thyroxine and triiodothyronine (about 0–10%) by liver slices (Fig. 1) or muscle homogenates (Table 4) was much less than the deiodination of thyroxine (about 75–90%) and probably less than the deiodination of triiodothyronine (about 30%). The deiodination of propionic acid analogues of thyroxine and triiodothyronine (30–45%) by liver slices (Fig. 1), though less than the deiodination of thyroxine (80%), was about the same as triiodothyronine, whereas deiodination of propionic acid analogues by liver and muscle homogenates (Table 4) was about the same as the acetic acid analogues. It is of interest that triiodothyropropionic acid was slightly more deiodinated than tetriiodothyropropionic acid (Fig. 1), the only triiodo form of thyro-

[3] β(5-hydroxy 2,4,6 triiodophenyl)-α-ethyl propionic acid.
[4] β(4-hydroxy 3,5 diiodophenyl)-α-phenyl propionic acid.
[5] β(3-amino 2,4,6 triiodophenyl)-α-ethyl propionic acid.

TABLE 3. DEIODINATION OF L-THYROXINE AND 1-3,5,3′-TRIIODOTHYRONINE BY RAT LIVER IN THE PRESENCE OF VARIOUS SUBSTANCES AT 10^{-3}M

Substance added	Mitochondria			Slices			Slices			Homogenate			Homogenate		
	% total thyroxine 6×10^{-8}M distributed as			% total thyroxine 7.1×10^{-7}M distributed as			% total triiodothyronine 6.5×10^{-7}M distributed as			% total thyroxine 7.1×10^{-7}M distributed as			% total triiodothyronine 6.5×10^{-7}M distributed as		
	T4	I^-	O	T4	I^-	O	T3	I^-	O	T4	I^-	O	T3	I^-	O
Control	10.6	77.5	11.9	13.3	80.2	6.5	56.3	40.7	3.0	25.0	65.0	10.0	49.2	40.2	10.8
$HgCl_2$	85.0	0.0*	5.0	80.6	9.2	10.2	77.8	16.1	6.1	67.5	28	4.2	77.9	17.8	4.3
$CuCl_2$	78.0	18.3	3.7	39.0	50.7	10.3	80.0	13.5	6.5	65.0	24.3	10.7	95.6	3.9	0.3
$CoSo_4$	68.4	15.7	15.9	42.3	47.0	10.7	75.4	20.0	4.6	73.0	14.5	12.5	87.4	8.4	4.2
Resorcinol	61.2	24.7	14.1	39.9	57.0	3.1	71.2	23.6	5.2	77.5	16.5	6.0	72.4	24.6	3.0
Estrone	82.4	9.2	8.4	48.0	41.6	10.4	92.1	6.8	1.1	76.5	20.0	3.5	94.5	5.5	0.0
Estradiol	—	—	—	56.1	34.3	9.6	94.5	5.5	0	78.5	13.0	8.5	93.6	6.4	0.0
Corticosterone	—	—	—	20.0	75.8	4.2	53.0	34.2	12.2	28.0	71.0	1.0	63.8	22.8	3.4
DOCA	—	—	—	21.4	72.9	5.7	57.4	40.7	1.9	—	—	—	60.7	30.9	8.4
Testosterone	—	—	—	5.2	85.7	9.1	64.2	25.9	9.9	33.0	61.5	5.5	67.5	27.9	4.6
Progesterone	—	—	—	8.6	89.6	1.8	68.3	25.5	6.2	27.5	66.0	6.5	64.2	32.9	2.9
$2CH_3$-1,4-Naphthoquinone	80.4	9.3	10.3	41.2	45.2	13.6	79.2	15.3	5.5	86.0	10.9	3.1	82.4	13.4	4.2
EDTA†	81.2	15.2	3.6	50.6	46.2	3.6	75.8	20.9	3.3	81.0	16.0	3.0	92.4	6.2	1.4
BHDB‡	24.9	57.2	17.9	80.7	10.2	7.1	93.2	3.8	3.0	—	—	—	—	—	—

* 10% of the radioactivity in the presence of $HgCl_2$ ran near the front. This was presumably iodide (10).
† Ethylene diamine tetraacetic acid.
‡ n-butyl parahydroxy 3,5 diiodobenzoate.

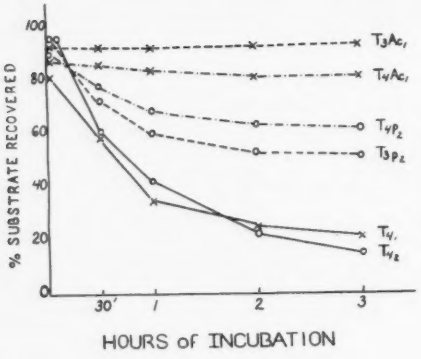

FIG. 1. The deiodination of the acetic and propionic acid analogues of thyroxine and triiodothyronine (10^{-8} *M*) by rat liver slices. The same subscript numbers indicate the same experiment. A different experiment (Table IV) indicated that there was slight deiodination of the acetic acid analogues.

nine studied to be deiodinated at least as easily and rapidly as the tetraiodo form.

Inhibition of deiodination by other iodinated thyronines and the acid analogues

Table 5 indicates that the deiodination of radiothyroxine and radiotriiodothyronine was inhibited by 10^{-4} *M* carrier thyroxine, triiodothyronine, and their acetic and propionic acid analogues. The deiodination of thyroxine was inhibited, in order of decreasing magnitude, by thyroxine, triiodothyronine, propionic acid analogues and acetic acid analogues. Triiodothyronine was inhibited about equally by all the iodinated thyronines tested though possibly less by the acetic and propionic acid analogues of thyroxine.

TABLE 4. DEGRADATION OF L-THYROXINE, TETRAIODOTHYROPROPIONIC AND ACETIC ACIDS, AND TRIIODOTHYROPROPIONIC AND ACETIC ACIDS BY RAT LIVER AND MUSCLE HOMOGENATE

	% total thyroxine 10^{-8}M distributed as			% total thyroxine 10^{-8}M distributed as		
	T4	I^-	O	T4	I^-	O
Control	97.9	2.1	0	80.6	16.6	2.8
Liver	47.4	42.9	9.7	—	—	—
Muscle	31.5	65.5	3.0	10.8	80.2	8.9
	% total tetraiodothyropropionic acid 10^{-8}M distributed as			% total tetraiodothyroacetic acid 10^{-8}M distributed as		
	T3PA	I^-	O	T4AC	I^-	O
Control	94.9	5.1	0	92.8	7.1	±
Liver	89.1	9.9	1	—	—	—
Muscle	83.8	14.7	1.5	83.4	13.5	3.2
	% total triiodothyropropionic acid 10^{-8}M distributed as			% total triiodothyroacetic acid 10^{-8}M distributed as		
	T4PA	I^-	O	T3AC	I^-	O
Control	91.2	8.3	0.5	95.4	4.6	0
Liver	82.3	15.9	1.8	—	—	—
Muscle	80.3	17.2	2.5	85.0	14.0	1.0

TABLE 5. DEGRADATION OF L-THYROXINE AND L-3,5,3′-TRIIODOTHYRONINE BY RAT LIVER SLICES IN THE PRESENCE OF VARIOUS IODOTHYRONINES

Substance added	Conc.	L-thyroxine 4.7×10^{-7}M			L-triiodothyronine 2.6×10^{-7}M		
		% of total distributed as			% of total distributed as		
		T4	I	O	T3	I	O
None		28.5	57.9	13.6	75.1	18.8	6.1
Triiodothyronine	3.2×10^{-4}	66.9	19.8	13.2	95.5	3.0	1.5
Thyroxine	2.0×10^{-4}	85.5	6.5	8.0	94.5	3.5	2.0
Triiodothyroacetic acid	3.1×10^{-4}	53.6	31.0	15.5	94.2	3.6	2.2
Tetraiodothyroacetic acid	3.0×10^{-4}	42.2	37.1	20.7	85.4	8.6	6.0
Triiodothyropropionic acid	3.3×10^{-4}	64.0	25.0	11.0	94.5	4.5	1.0
Tetraiodothyropropionic acid	3.4×10^{-4}	61.8	22.8	15.4	87.9	7.4	4.7

Deiodination of endogenously labelled thyroxine

The incubation of endogenously labelled thyroxine with rat liver slices resulted in the formation of radioiodide. No material with the R_f of 3,5 diiodothyronine or any other new compound was identified chromatographically. A small amount of a compound could have gone undetected because there was little radioactivity, but in the five solvent systems used no new material of a significant amount, relative to iodide and thyroxine, was noted.

DISCUSSION

The liver, kidney and muscle (but not the thyroid (10, 11)) of rats deiodinated radiothyroxine and radiotriiodothyronine and, to a certain extent, their acetic and propionic acid analogues. This system had an optimal temperature (although the present study confirms the relative heat stability of the homogenate (12)), required oxygen, was inhibited by boiling, certain toxic compounds (some heavy metals, phenols, quinone, cyanide) estrogenic substances and BHDB. It was strikingly unaffected by many antithyroid compounds, other iodinated compounds and a sulphydryl inhibitor (p-chloromercuribenzoate).

The present finding of inhibition of deiodination of thyroxine by estrogenic substances is in contrast to that of Cruchand *et al.* (13). These authors, however, used estradiol monophosphate in a smaller concentration. Such an inhibition might be an additional cause for the rise in blood levels of thyroxine in humans with high estrogen levels. *In vivo* human studies, as yet incomplete, suggest, in several instances, the possibility of a block by stilbesterol of the deiodination of thyroxine.

The main product of this deiodinating system was radioiodide. The absence of radiotriiodothyronine, in spite of its being less rapidly deiodinated than thyroxine, suggests that it was not formed. The same reasoning applies to the acetic and propionic acid analogues. The substrates were labelled only in the prime positions and therefore 3,5 diiodothyronine or a

derivative thereof, would have gone undetected. No 3,5 diiodothyronine was found, however, in the deiodination of endogenously labelled thyroxine but, since we do not know how rapidly 3,5 diiodothyronine may be deiodinated, we cannot conclude that it was not formed. The finding that 3,5 diiodothyronine did not inhibit the deiodination of thyroxine suggests that it was not or, if so, only slightly deiodinated by the same enzyme system. In addition, 3,5 diiodothyronine should be more difficult to deiodinate than thyroxine. Of the two possibilities, 3,5 diiodothyronine was not formed during the deiodination of thyroxine or 3,5 diiodothyronine was formed but was rapidly metabolized, the former would, therefore, seem the more likely. As previously noted, the radioactivity was small and the possibility of 3,5 diiodothyronine derivatives or other compounds being formed in small quantities cannot be ruled out. These results suggest that either a compound was formed with the chromatographic characteristics, in five different solvent systems, of thyroxine or iodide, or thyroxine was deiodinated, at least to a major extent, to iodide and thyronine (or some completely deiodinated thyronine derivative).

Others have found an accumulation of radiotriiodothyronine (1, 2, 3 13), acetic acid analogues (3, 5, 6) propionic acid analogues (3), pyruvic acid analogues (7, 8) and glucuronide acid conjugate (9) from radiothyroxine metabolized either *in vivo* or *in vitro*. In the present study deiodination by homogenates was resistant to boiling compared to deiodination by slices or mitochondria and BHDB inhibited deiodination by slices much more than deiodination by mitochondria. There may, therefore, be several enzyme systems (deiodinating, deaminating, decarboxylating) capable of metabolizing thyroxine and the results may vary with the tissue studied and the method of study.

The question of where the deiodinating enzyme system is remains unresolved. Mitochondria seemed particularly effective in the system studied here. A deiodinating system at the cell surface, however, might serve to deiodinate thyroxine to facilitate its entrance into the cell and might explain why thyroxine is more rapidly deiodinated than triiodothyronine. There was no evidence in the present study, however, for the formation of any iodinated thyronine by the deiodination of thyroxine; nor was there any evidence to indicate whether the deiodinating system acted within or on the surface of the cell in the intact tissue. Furthermore, deiodination may have no relation, directly, to the metabolic activity of the thyroid hormone, in spite of the fact that BHDB both reduces oxygen consumption and inhibits deiodination (12).

The deiodinating system presently under consideration seems specific principally for tetra and triiodinated thyronine compounds. The addition of tetra- and tri-iodinated thyronines inhibited the deiodination of thyroxine or triiodothyronine to an extent often commensurate with the amount

of deiodination which these compounds underwent when added alone to a similar system. 3,5 Diiodothyronine did not inhibit deiodination of thyroxine, nor did the iodinated tyrosines, iodide, Priodax, Telepaque or Teridax. It is of interest, however, that an iodinated single benzene-ring compound (BHDB) did inhibit deiodination suggesting that it may be deiodinated itself, and indicating a lack of discrete specificity for the enzyme system involved.

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THE METABOLISM OF I^{131}-LABELED THYROXINE, TRIIODOTHYRONINE AND DIIODOTYROSINE BY AN ISOLATED PERFUSED RABBIT LIVER[1]

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ABSTRACT

Isolated rabbit livers were perfused for 12 to 15 hours with I^{131}-labeled thyroxine, triiodothyronine and diiodotyrosine in order to evaluate the hepatic metabolism of these substances. The livers were able to concentrate these amino acids in appreciable quantities showing somewhat greater accumulation in livers perfused with labeled triiodothyronine than with thyroxine. Triiodothyronine and thyroxine were metabolized by the perfused liver at rates identical in ratio to the intact rabbit but faster by a factor of almost six. Diiodotyrosine, however, was deiodinated at a rate similar to that in the intact animal. Measurements of biliary excretion of radioactivity showed that this pathway proved more important over the period studied for livers perfused with triiodothyronine than for those perfused with thyroxine. Twenty-six per cent of initial radioactivity was excreted over 12 hours in the bile of the former and 8% in the latter. Less than 3% of the initial perfusate radioactivity appeared in the bile and over 12 hours when perfusion with labeled diiodotyrosine was carried out.

The perfused liver proved capable of producing triiodothyronine by the deiodination of thyroxine in the majority of studies. Several unidentified compounds appeared in both the bile and the plasma of the perfusate following perfusion with both thyroxine and triiodothyronine. It is likely that the most abundant of these components is a glucuronide conjugate of thyroxine and triiodothyronine.

THE metabolism of thyroid hormone by the liver has been a matter of considerable interest since 1919 when Kendall (1) showed that circulating thyroxine is partly collected by the liver and that derivative iodides can be recovered in the bile. Using radioisotopic and chromatographic techniques, many recent investigators have restudied this subject. Information, however, is still lacking as to the precise role played by the liver in the peripheral metabolism of thyroid hormones.

The importance of the enterohepatic circulation in the metabolism of thyroxine has been emphasized by Albert and Keating (2) and by Myant (3). There is considerable evidence that the liver plays a key role in the regulation of the thyroid hormone content of the body by destruction of excess circulating hormone and regulation of biliary excretion mechanisms. Several workers have investigated the nature of thyroid hormone deriva-

[1] This work was carried out in part at the Surgical Research Unit, Brooke Army Medical Center, Fort Sam Houston, Texas. Presented at the 1958 Meeting of the American Goiter Association.

ives in bile, and the importance of the glucuronide conjugation mechanism n the metabolism of thyroxine has been well established (4, 5, 6, 7, 8). In ıddition to glucuronides, recent work (9) has shown that the iodothyro- ɔyruvic acid derivatives of both thyroxine and triiodothyronine may be ound in the bile of rats.

The site of origin of triiodothyronine in the plasma has not been estab- ished. There is appreciable evidence that it may arise from the deiodina- ion of thyroxine (7, 10, 11, 12, 13, 14) although agreement is not unani- nous (15). *In vitro* studies (16, 17, 18, 19, 20) indicate that the liver may ɔe one of the sites of peripheral deiodination.

Using a perfusion apparatus, it was felt that it might be possible to iso- ıte the action of the liver in the metabolism of thyroid hormones and to investigate its function, not only as part of an excretion mechanism, but for its possible importance in the deiodination of these substances.

METHODS

The perfusion technique used in these studies was described previously by Young, Prudden and Stirman (21). In this procedure, the liver of an anesthetized adult New Zealand rabbit was carefully removed following cannulation of the portal vein, inferior vena cava, and the bile duct with polyethylene catheters. The liver, which weighed about 100 grams, was placed in the apparatus with the bile duct cannula emptying into a collection tube. The perfusate was a 50–50 mixture of defibrinated, heparinized rabbit blood and Hartman's solution with a total volume of 1000 cc. It was oxygenated by permitting it to flow freely from the vena cava through a Vigreaux fractionating column connected to an oxygen source. After collection in a mixing chamber the perfusate was then pumped to a hydrostatic reservoir from which it flowed into the liver through the portal vein at a rate of about 150 cc. per minute. The apparatus was kept in a thermostatically controlled box at a temperature of 37° C. In order to avoid bacterial contamination, the entire apparatus was cleaned and autoclaved, and sterile technique was used in the preparation of the perfusate. In addition, Aureomycin was added to the perfusate at four hour intervals during the procedure. The average liver was perfused for a period of 12 to 15 hours.

Commercially prepared I^{131}-labeled *l*-thyroxine, l-3,5,3′-triiodothyronine and diiodotyrosine,[2] in amounts of 1 to 10 micrograms containing from 30 to 150 microcuries of I^{131} were used as the substrate. This material as received showed various degrees of impurity and was purified by paper chromatography before use. In several of the procedures, the starting thyroxine was contaminated with a small amount of triiodothyronine and iodide despite purification. The labeled amino acid was thoroughly mixed with the perfusate which was then added to the apparatus. A control sample of perfusate was taken before the liver was placed in the apparatus, a second sample taken immediately after the start of perfusion of the liver and a third 5 minutes later. Additional samples were taken every 30 minutes for the first two hours and hourly thereafter.

The radioactivity of the plasma of the perfusate and of the bile was determined by measurements in a scintillation well counter. The proteins of the identical plasma aliquot were precipitated using 5 volumes of 20% trichloracetic acid. The precipitate was washed twice with 5% trichloracetic acid and then measured in the well counter to determine the protein-bound I^{131} (PBI^{131}).

Measurements of total protein, albumin (22) and urea nitrogen (23) of the perfusate

[2] Obtained from Abbott Laboratories, Oak Ridge, Tenn.

were made every two hours. Samples of the perfusate for chromatography were obtained before the liver was added to the apparatus and every three hours thereafter. When bile flow permitted, samples taken at similar intervals were used in the examination of the bile.

Samples of the plasma of the perfusate and the bile were prepared for chromatography by acidifying with 0.2 cc. of 5N HCl per cc. of sample and then extracting twice with 5 cc. of *n*-butanol (24). The supernatants were combined and reduced to dryness by vacuum distillation at room temperature. The residual was dissolved in a small amount of butanol and chromatographed in an ascending butanol-dioxane-ammonia system (25). The dioxane was purified by distillation. Selected samples were chromatographed, in addition, in an ascending butanol-acetic acid system (26). Appropriate carriers were applied to all chromatograms before development and were identified with ninhydrin or sulfanilic acid (27). All chromatograms were radioautographed on no-screen X-ray film and then scanned with an automatic geiger counter and recording rate-meter. Radioautographic spots were identified by comparison of the film with the location and shape of the carrier spot on the chromatogram. The radioactivity on the chromatogram was quantitated by planimetry of the rate-meter recording after identification and delineation of each peak by comparison with the corresponding radioautograph.

RESULTS

Seven technically satisfactory perfusions with thyroxine, four with triiodothyronine and one with diiodotyrosine were completed. Over the course of the average procedure the pH of the perfusate remained relatively constant. Measurement of albumin and globulin showed little change. The urea nitrogen accumulated according to a single exponential cumulative equation and doubled by the end of 12 hours. This pattern of urea increase was taken to indicate the presence of actively functioning hepatic tissue. The mean rate constant of the expression for urea accumulation for those livers perfused with thyroxine was 0.00181 min.$^{-1}$. This represents a half-life of accumulation of 383 minutes. For those livers perfused with triiodothyronine, the same formulation showed a mean rate constant of 0.00118 min.$^{-1}$ (half-life = 587 min). When the difference between these mean values was examined by "t" test, it was found, interestingly enough, that the difference was significant ($P = 0.01$). Hence urea nitrogen accumulated significantly more rapidly in those livers perfused with thyroxine compared with those perfused with triiodothyronine. The physiological implication of this finding is not immediately apparent.

Control procedures included circulation of the perfusate with its labeled compound for twelve hours in the apparatus without a liver. In these control studies, the total radioactivity, protein-bound I^{131} and chromatograms all showed no significant change from the starting determinations. During the course of those control studies without the liver, measurement of the perfusate urea nitrogen (which was essentially constant) showed a standard error of 1.5% of the mean perfusate value. Similarly, measurement of the PBI^{131} showed a standard error of the hourly measurement of 0.27% of the mean. The measurement of total plasma radioactivity showed a standard error of 0.83%. These figures represent the intrinsic errors of the proce-

dures and are considered satisfactory. Perfusion of sodium radioiodide through a liver for the same period showed no changes in the indices studied. The iodide was excreted rapidly in the bile. Over the course of the control experiments there was a loss of radioactivity (presumably adsorbed to glassware) equivalent to 0.75% of the total activity per hour.

RATE STUDIES

Perfusate

The total I^{131} of the perfusate showed a rapid decrease over the first two hours. At the end of two hours the livers perfused with thyroxine had accumulated a mean of 24% while those perfused with triiodothyronine had accumulated a mean of 39% of total radioactivity. This difference was statistically significant ("t" test, P·0.01). The total perfusate activity in all studies then remained at a fairly constant level for the duration of the procedure. Since the system is a closed one, this first phase of disappearance of radioactivity was interpreted as indicating either passive or active distribution of radioactivity within the liver.

Measurements of the PBI^{131} of the plasma of the perfusate showed that triiodothyronine was deiodinated at a significantly faster rate than was thyroxine (Fig. 1). The mean half-life for the disappearance of protein-bound I^{131} when the liver was perfused with thyroxine was 32.5 hours while that for triiodothyronine was 22.2 hours. This difference is significant. The single satisfactory procedure in which the liver was perfused with diiodotyrosine showed an appreciably different and much faster rate of deiodination (half-life, 9.4 hours).

By contrast, measurements of the relative radioactivity of each peak appearing on the chromatograms permitted the estimation of the disap-

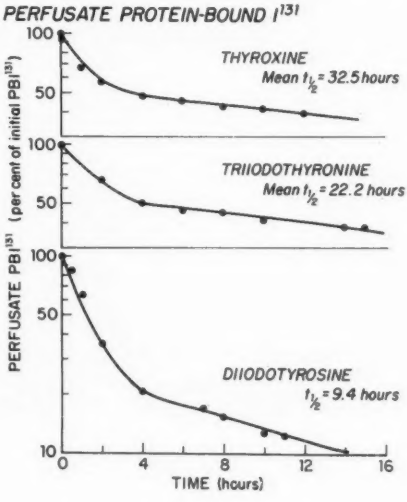

FIG. 1. The disappearance of the perfusate protein-bound I^{131} for livers perfused with I^{131}-labeled thyroxine, triiothyronine and diiodotyrosine. The half-lives for thyroxine and triiodothyronine are the means of 7 and 4 experiments respectively, while that for diiodotyrosine represents a single study. The ordinate represents the logarithm of the perfusate PBI^{131} while the abscissa represents time in hours.

pearance rates for chromatographically pure thyroxine and triiodothyronine. Here, the thyroxine disappeared from the perfusate with a mean half-life of 24.3 hours while triiodothyronine was removed at a significantly faster rate with a mean half-life of 9.5 hours. The disappearance rates, calculated in this fashion, were thus somewhat faster than when calculated from the determinations of the perfusate protein-bound I^{131} (see above).

Analysis of the perfusion data for the kinetic rate constants shows that the specific rate of liver degradation for thyroxine or triiodothyronine is directly related to the weight of the liver. The rate constant "k" (% degraded per hour per 100 gm. of liver) is 3.8% for thyroxine and 8.0% for triiodothyronine. Further calculation indicates an average degradation of 1 to 2 μg. of thyroxine per 100 gm. of liver for a twelve hour period.[3]

Bile

Over a period of twelve hours, those livers perfused with thyroxine excreted in the bile a mean of 8% of the total radioactivity initially added to the perfusate. However, radioactivity derived from triiodothyronine was found to have a total biliary excretion over the same period of a mean of 26% of total radioactivity. Thus despite equality of bile production (averaging 2 to 6 cc. an hour) with both amino acids, the perfused liver excreted in the bile three times as much radioactivity derived from triiodothyronine as compared to that derived from thyroxine. The biliary excretion curves of radioactivity for thyroxine perfused livers showed an early peak of radioactivity (three to four hours) followed by a steady fall. Following labeled triiodothyronine administration however, radioactivity was excreted in the bile with a different pattern, showing a slow rise in hourly excretion reaching a peak by 7 to 8 hours and then falling off slightly.

In the liver perfused with labeled diiodotyrosine, a total of only 2.4% of the total perfusate radioactivity appeared in the bile over a 12 hour period.

CHROMATOGRAPHIC STUDIES

Perfusion with I^{131}-labeled thyroxine

In those livers perfused with labeled thyroxine, chromatographic studies of the perfusate showed that thyroxine gradually decreased with time while iodide, several unknown materials and triiodothyronine appeared (Fig. 2). Similarly, chromatograms of the bile showed all of these compounds (Fig. 3).

The relative quantities of radioactivity in each compound separated on the chromatograms of the perfusate for a typical liver perfused with I^{131}-labeled thyroxine are shown in Figure 4. Thyroxine decreased in a moderately slow and irregular fashion with a drop over 12 hours to a mean of 48% of initial radioactivity. Triiodothyronine appeared in the perfusate

[3] The kinetic formulation used in these calculations will be presented in a subsequent publication.

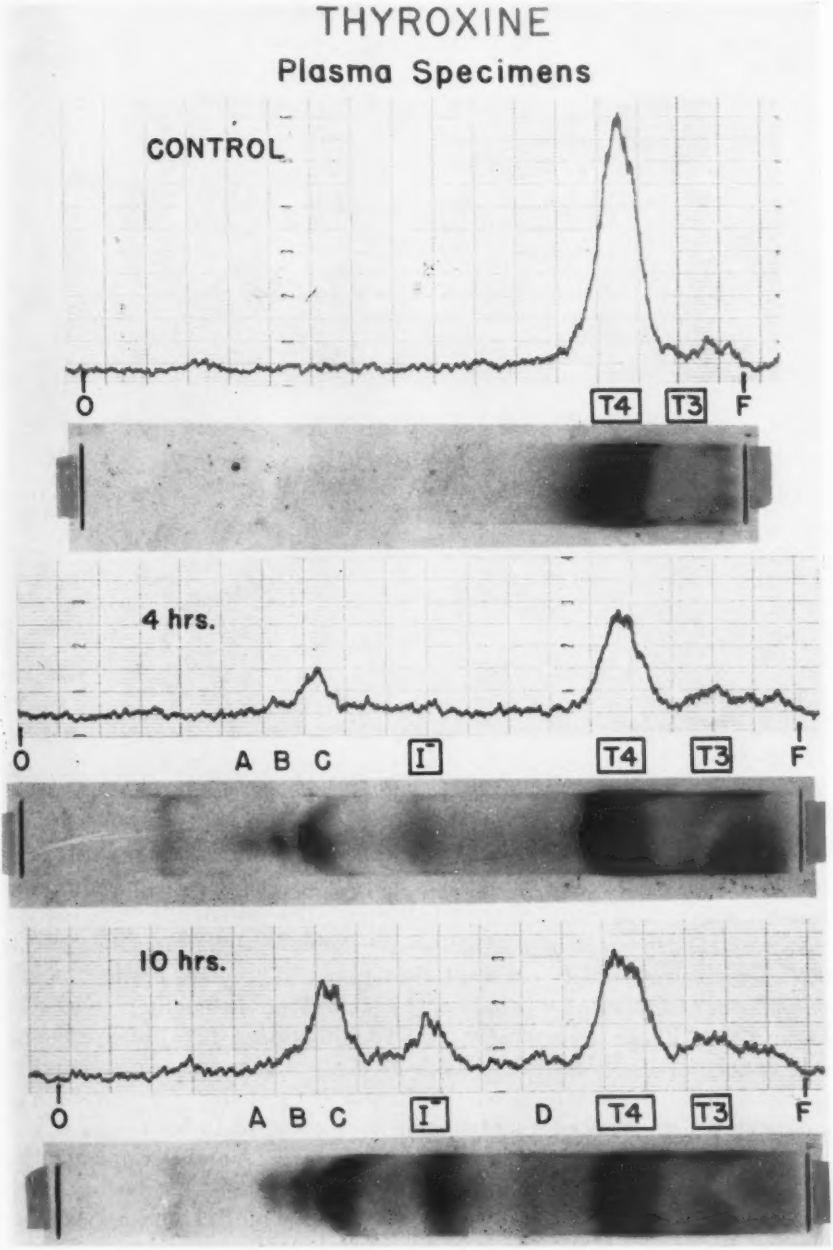

FIG. 2. Perfusion of a liver with I^{131}-labeled thyroxine. The radioautographs of chromatograms of the plasma of the perfusate are placed beneath the corresponding rate-meter recordings of radioactivity content. Legend: O = origin, F = front, T4 = thyroxine, T3 = triiodothyronine, I^- = iodide. The unknown peaks are labeled A, B, C, and D.

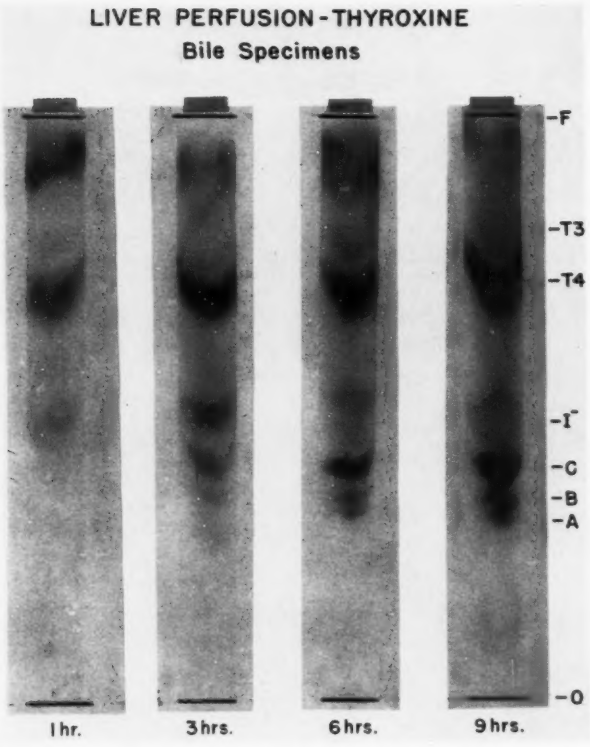

FIG. 3. Perfusion of a liver with I^{131}-labeled thyroxine. Radioautographs of bile samples obtained 1, 3, 6, and 9 hours after the start of perfusion. The legend is the same as in Fig. 2.

in significantly increased quantities in four of the seven procedures in which the liver was perfused with thyroxine. The remaining three started with appreciable quantities of triiodothyronine and the area under this peak remained relatively constant throughout. The main product of the breakdown of thyroxine was iodide which slowly and steadily increased to reach its maximum concentration of 8 to 25% (mean 15%) of total chromatogram activity by 12 hours (Table 1). Several unknown compounds appeared and the predominant one, Unknown C (probably a glucuronide as indicated below) reached a mean value by the end of 12 hours of 7.2% of total chromatogram activity. Two other unknowns, A and B, appeared less constantly and in smaller quantities. Unknown B usually reached its maximum concentration of 2 to 3% by the sixth to the ninth hour and then decreased. Unknown A was found in the plasma on two occasions, and Unknown D was seen only once.

Analysis of the chromatograms of the bile for a typical liver perfused

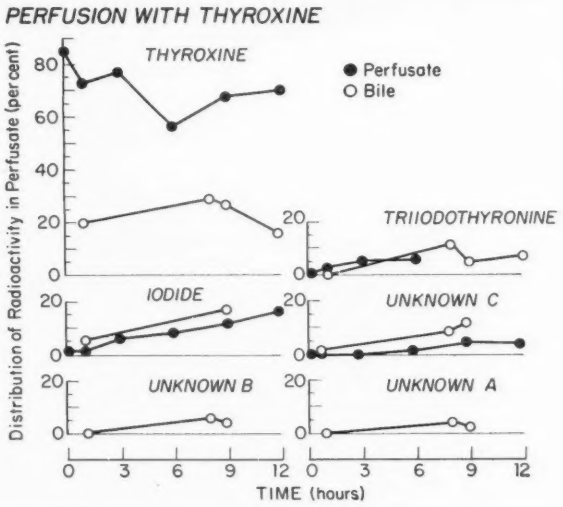

FIG. 4. Distribution of the relative radioactivity content of chromatograms of the perfusate (solid circles) and bile (open circles) of a liver perfused with I^{131}-labeled thyroxine. The ordinate represents the percent of the total chromatogram radioactivity while the abscissa represents the time of the samples studied.

with labeled thyroxine (Fig. 4) showed that thyroxine was the major component as early as thirty minutes after perfusion was started at which time the first bile specimen was examined. The thyroxine content of bile over the period of twelve hours remained relatively constant. Triiodothyronine and iodide appeared in all bile specimens in about the same concentration that was seen in the plasma (in terms of total activity of the chromatogram). Unknown C not only appeared in the bile much earlier than in the perfusate, but was usually present there in much greater concentration (Table 1). At the end of 12 hours Unknowns A and B were found in the bile in all thyroxine perfusions with respective mean concentrations of 4.3% and 9.6% of total chromatogram activity.

TABLE 1. DISTRIBUTION OF CHROMATOGRAM RADIOACTIVITY

(All values are means; they are expressed as per cent of the total chromatogram radioactivity upon termination of perfusion.)

Perfusate	Specimen	Thyroxine	Triiodothyronine	Iodide	A	B	C	D
I^{131}-thyroxine	perfusate	48	5.8	15	—	—	7.2	—
	bile	24	5.9	16	4.3	9.6	19	—
I^{131}-triiodothyronine	perfusate	4.4*	21	14	2.7	11	22	7.0
	bile	7.0	15	11	3.6	4.9	38	8.8

* Contaminant of the triiodothyronine initially added to perfusate.

Perfusion with I^{131}-labeled triiodothyronine

Chromatographic studies of the perfusate of those livers perfused with labeled triiodothyronine showed qualitatively similar results to those described above for the thyroxine. Similar measurements of the relative content of the substances found in the perfusate and the bile are shown during the course of a typical perfusion in Figure 5. In the perfusate, chromatography of the control specimens showed that a mean of 83% of the initial total perfusate radioactivity was present as triiodothyronine. After twelve hours, however, the amount of triiodothyronine had dropped sharply to

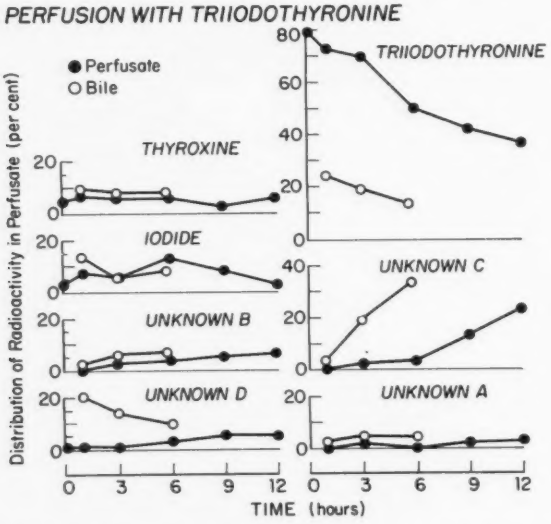

FIG. 5. Distribution of the relative radioactivity content of chromatograms of the perfusate and bile of a liver perfused with I^{131}-labeled triiodothyronine. The ordinate and abscissa are identical with that in Fig. 4.

21% of initial radioactivity. In almost all procedures where I^{131}-triiodothyronine was added to the perfusate, thyroxine in quantities of 5 to 10% was present as an initial contaminant. Unknown C with a mean perfusate concentration of 22% by the end of 12 hours was present in much greater concentration than was seen when thyroxine was the starting material. Iodide made up about 14% of the total radioactivity and Unknown B was present in rather high concentrations (mean 11%). Unknown A was found in all plasma samples with a mean concentration at the termination of the perfusion of 2.7% of the total chromatogram activity (Table 1).

Examination of the bile showed that the major component was Unknown C. This compound, in those livers perfused with triiodothyronine, contributed in 12 hours more than one third of the total biliary activity; an amount twice as great as that found following perfusion with thyroxine.

Unknowns A and B were present in all late bile samples but in relatively small quantities. Unknown D was found in all specimens of bile and was the only derivative compound that appeared initially (one hour) in higher concentrations (16%) than was seen after twelve hours of perfusion.

Hydrolysis with β-glucuronidase of Unknown C eluted from the chromatogram yielded a substance that traveled on the chromatogram to the same location as carrier triiodothyronine.

Perfusion with I^{131}-labeled diiodotyrosine

In those livers perfused with labeled diiodotyrosine, chromatograms showed the rapid disappearance of diiodotyrosine from the perfusate concomitantly with the appearance of iodide. Before perfusion started, 97% of the total radioactivity of the chromatogram was present as diiodotyrosine. However, iodide appeared rapidly in the perfusate with a concentration by the end of the 2nd hour of 23%, by the end of the 6th hour of 40% and by the end of the 12th hour of 46% of chromatogram radioactivity. Examination of the bile showed the early presence of both diiodotyrosine and iodide but as perfusion continued, the former decreased to minimal quantities while the latter accounted for almost all of the radioactivity of the chromatogram.

DISCUSSION

The viability of the liver in the perfusion preparation is reasonably assured by the findings of the rising urea nitrogen of the perfusate, maintenance of total protein levels, and continued production of bile in appreciable quantities. Microscopic examination of the liver upon termination of each study showed intact morphology. Only those perfusion studies that met the above criteria were considered to be satisfactory and included in the series.

It is interesting to note that the relative rates of deiodination of thyroxine and triiodothyronine by an isolated perfused liver are roughly analogous to those obtained in intact animals where triiodothyronine is not only removed from the blood more rapidly than thyroxine but is deiodinated to a greater degree (28, 29, 30). *In vivo* studies in rabbits of the same breed used in these perfusion experiments showed that the average half-time of disappearance of plasma radioactivity after administration of labeled thyroxine was 192 hours, that for triiodothyronine 125 hours and that for diiodotyrosine 14.5 hours. (31) The disappearance rates of the protein-bound I^{131} obtained by liver perfusion (32.5 and 22.2 hours respectively) are faster by a factor of almost 6 than those seen for both thyroxine and triiodothyronine in the intact animal. It is striking to note that the ratio of the degredation half-time of thyroxine compared with that for triiodothyronine is virtually identical for both the perfusion preparation and the intact animal. However, for the liver perfused with diiodotyrosine, the deiodination rate is very similar to that in the intact animal (9.4 and 14.5

hours, respectively). This is of interest in view of Flock's (7) observation that the rate of deiodination of diiodotyrosine was not altered by evisceration of the rat. The rate of deiodination of both thyroxine and triiodothyronine was reduced in the eviscerated rat over that seen in the intact animal although triiodothyronine continued to disappear from the blood more rapidly than thyroxine. *In vitro* studies (32) indicate that the liver readily metabolizes diiodotyrosine; however Flock's observations and those of the present study suggest that the liver may not play a major role in the deiodination of diiodotyrosine in the intact animal.

In the present study, a greater role by a factor of three was played by the bile of those livers perfused with triiodothyronine than in those perfused with thyroxine in terms of the amount of total radioactivity excreted. Cooper and Beringer (33) noted no significant difference in the biliary excretion of radioactivity following administration of labeled thyroxine and triiodothyronine to bile fistula dogs, finding one-third of the administered dose in the bile by 120 hours. Roche, however, noted that 60% of radioactivity derived from injected I^{131}-labeled triiodothyronine appeared in the bile of rats by 15 hours compared to 40% of that derived from I^{131}-labeled thyroxine.[4]

The chromatographic findings of triiodothyronine in the perfusate and the bile of livers perfused with labeled thyroxine suggest the ability of the liver to deiodinate thyroxine. *In vitro* studies by several workers have shown that liver tissues have the capability of deiodinating thyroxine (16, 17, 18, 19, 20) and the existence of deiodinase systems in the liver has been demonstrated by others (34, 35). In addition, Flock (8) has shown that the hepatectomized dog does not deiodinate thyroxine and triiodothyronine as completely as the normal animal. The present findings therefore add further evidence for the existence of a peripheral mechanism for the formation of triiodothyronine from thyroxine and suggest that the liver may play an important role in this function.

Of the unknown compounds noted in the perfusate and bile in the present experiments, there is suggestive evidence that the major one (Unknown C) is a glucuronide. Briggs *et al.* (4) in a study in which a rat liver was perfused with labeled thyroxine found in the bile appreciable quantities of a substance subsequently identified as the glucuronide conjugate of thyroxine (5). Roche *et al.* (9) and Flock and Bollman (7) identified this substance in bile.

The presence of thyroxine glucuronides in the plasma has been described only in animals whose bile ducts have been ligated (37) or whose livers have been severely damaged (20). In the present studies a compound that was probably a glucuronide was found in appreciable quantitites in the

[4] Data presented by Roche during the discussion (p. 224) of a paper by Vannotti (20).

perfusate following perfusion of livers with thyroxine and triiodothyronine. Its presence may indicate undetected biliary damage. More likely, however, since the perfusion takes place in a closed system, it is the result of the absence of a urinary excretion mechanism which could handle the rapid removal of this material from the plasma.

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RELATION OF NUCLEIC ACIDS TO THE STRUCTURE AND FUNCTION OF THE GUINEA PIG THYROID GLAND

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ABSTRACT

Groups of guinea pigs were administered thyrotropin, thyroxine, and thiouracil. The weight, functional activity (I^{131} uptake), relative percentages of tissue constituents, average cell height and the cell population density of the thyroid glands were determined. Desoxyribonucleic acid and ribonucleic acid of thyroid tissue were determined by chemical methods. Since the histological structure of the thyroid gland is a variable factor, it should be considered in the interpretation of data obtained by chemical analysis of whole tissue samples. A method is described for expressing the results of chemical analyses of thyroid gland nucleic acids in terms of a calculated thyroid parenchymal cell mass. These results differ in significance from the conventionally expressed values based on a unit weight of whole tissue sample. The nuclear content of DNA was found to be independent from the stimulating action of thyrotropin. In the thyrotropin stimulated gland, an increase of RNA was observed. In the absence of thyrotropin stimulation, a decrease in RNA occurred. Under the stimulation of thyrotropin, the increase in total RNA was proportionally greater than the increase in average weight of the gland. The increase of total RNA per gland was less marked when related to the increase in total cell mass of the gland. In the absence of thyrotropin stimulation (thyroxine-injected animals), the decrease in total RNA per gland was proportionally greater than the decrease in average weight of the gland; but this effect was more pronounced when the decrease in total RNA was related to the decrease in total cell mass of the gland. Thiouracil *per se* probably does not inhibit the synthesis of RNA in the thyroid cell.

THERE is an abundant literature on the nucleic acids of most mammalian organs but relatively little concerning the thyroid gland. Nucleic acids of the thyroid have been determined by histochemical (1, 2), histospectroscopic (3, 4), and chemical methods (5, 6, 7, 8). The results of histospectroscopic studies are expressed in arbitrary units, while the data of chemical analyses are usually reported as concentration per unit of wet weight of tissue or content per whole gland.

The structure of the thyroid gland makes it difficult to interpret chemical assays if expressed in terms of the weight of whole tissue. Since the thyroid is not a solid organ, but contains follicular spaces which constitute

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a large and variable factor, it might prove helpful to express the results of chemical analyses in terms of a calculated cell mass which excludes stromal elements and colloid. This paper is concerned with an histological and chemical technique which has provided a measurement of the nucleic acids per unit weight of thyroid protoplasm. The method has been utilized in studies of the effects of thyrotropin, thyroxine, and thiouracil upon the metabolism of nucleic acids in the guinea pig thyroid gland.

METHODS

Male guinea pigs weighing 165–210 gm. were used in these experiments. Each group consisted of five animals fed *ad libitum* with a regular diet and lettuce. Control and experimental groups of animals were sacrificed after 14 days and after 3 months. One group received 0.5 U.S.P. units of thyrotropin (Armour) in 0.5 ml. saline subcutaneously daily for 14 days. One group received 100 μg. of *l*-sodium thyroxine in 0.5 ml. saline (pH 8.5) daily subcutaneously for 14 days. One group was fed with 0.2% thiouracil ground in food for 3 months. The weight of all animals was regularly recorded. Animals were sacrificed by a blow on the head and exsanguination. Eight hours before being sacrificed they were injected with 0.5 μc. I^{131} in 0.5 ml. saline subcutaneously. The food was not omitted until the end of the experiment. The I^{131} in each thyroid gland was counted in a well-type scintillation detector, and counts were expressed as per cent of administered dose. Aliquots of thyroid tissue of thiouracil-fed animals were also precipitated with 20% trichloracetic acid and then counted in a well-type scintillation detector. Homogenized aliquots of these thyroids were digested with trypsin and chymotrypsin and thereafter were chromatographed in a butanol-2 N acetic acid solvent system. Strips were counted in segments in a well type scintillation detector. The thyrotropin and thyroxine-treated animals gained weight during 7 days of the control period. In the experimental period, despite increased food intake, the thyrotropin and thyroxine-treated animals lost $\frac{1}{6}$ and $\frac{2}{3}$ respectively of the weight which they had gained during the control period. Both groups, especially the thyroxine-treated animals, had evidence of hypermetabolism. Control groups and thiouracil-fed animals gained weight throughout the experiment. The thiouracil-fed animals did not develop signs of hypometabolism.

Histological Methods

A calculated cell mass value may be obtained by an histometrical determination of the relative percentages of thyroid tissue constituents. One medial part of each guinea pig thyroid lobe was fixed in 10% formalin, routinely processed and embedded in paraffin. Sections of 6μ thickness were cut from the median portion and stained by hematoxylin and eosin, Feulgen, and Masson tri-chrome techniques. Quantitative estimates of the relative percentages of thyroid tissue components were made by the method of Chalkley (9). This consists of counting a series of random tissue contacts in a number of high power microscopic fields made by points fixed in the microscope eye-piece. A ratio is thus obtained by comparing the total number of random ocular point intersections for each tissue constituent. Parenchymal thyroid cells, follicle spaces, and stroma were the tissue fractions studied. Homogeneity of tissue architecture is desirable for a reasonably accurate application of this sampling technique. The thyroids were quite uniform in structure, as was demonstrated by obtaining similar results on tissue composition from sections cut at three different levels of a thyroid lobe in the experiment with thiouracil-fed animals.

Three hundred random tissue point-intersections were made in an average total of 25 high-power fields per section. Point-contacts with the lumens of vessels were con-

idered as part of the stroma, but where the random ocular points coincided with an mpty tissue space (other than intrafollicular or intravascular in position), these were mitted as being tissue artifacts. The Masson tri-chrome stain greatly facilitated the tudies by defining connective tissue elements from parenchymal cells.

By assuming an approximate equivalent specific weight of parenchymal cell, follicle pace fluid, and stromal elements, the percentages of relative tissue components by area ere translated into similar porportions on the basis of total gland wet weight. For xample, if a gland of 100 mgm. had 35% parenchymal cells, 45% follicle spaces, and 0% stroma by histological cross-sectioned area calculation, it was assumed that the elative weight fractions were 35 mgm., 45 mgm., and 20 mgm. respectively. Some icense with accuracy was taken in equating the area percentages of tissue constituents ith volume and weight, but the differences in the specific weights of the various tissue omponents were regarded as insignificant. Since the values are relative and subject to similar artifacts (i.e., shrinkage), these errors were considered to be relatively small.

The total thyroid cell mass per whole gland was then defined as the product of the ercentage of thyroid cells per gland and the total wet weight of the gland. Therefore, he RNA concentration per unit weight of cell mass (RNA μg./mgm. thyroid cell mass) an be computed as follows:

$$\frac{\text{RNA } (\mu\text{g./mgm. gland}) \times \text{total gland weight (mgm.)}}{\text{Fraction of gland as cells} \times \text{total gland weight (mgm.)}}$$

$$= \frac{\text{Total RNA } (\mu\text{g.})/\text{thyroid gland}}{\text{Total thyroid cell mass (mgm.)}} = \text{RNA } (\mu\text{g.})/(\text{mgm.}) \text{ thyroid cell mass.}$$

In order to obtain an index of the actual number of cells in thyroid tissue, estimates of relative thyroid cell population density were made by counting the number of thyroid cell nuclei in a standard area of gland median section, using an ocular grating ruled into squares (10). Three separate counts of the nuclei lying within a standard area ($84{,}500\mu^2$) were made on each section (total area, $253{,}000\mu^2$). The average of these values was recorded as the "cell population density factor," or the number of thyroid cell nuclei per $84{,}500\mu^2$ area. This index constitutes an estimate of cell population density of an entire gland and represents a fair sampling of a central thyroid lobe section. As was observed in comparing sections cut at three different levels of one series, there was a remarkable degree of homogeneity of the tissue. The average nuclear size remained quite constant in the various experiments and was therefore not considered a significant variable in these cell population calculations.

Average thyroid cell height measurements were obtained by the ocular micrometer direct technique using oil immersion objectives. A total of twenty-five representative cells, each from one randomly selected intact follicle, was measured in each gland section (11).

Since the structure of the thyroid makes technically difficult an exact isolation for counting of nuclei from tissue homogenates, the average DNA content in the guinea pig diploid nucleus (5.9×10^{-12} g.) was used in calculating the number of cells per gland (12). This value represents the average DNA content of one nucleus from resting tissue with a low mitotic index, so that calculations on more actively dividing thyroid cells (i.e., thyrotropin-treated animals) have only relative significance. The total number of cells in the thyroid was determined by dividing the DNA per whole gland by the assumed average nuclear DNA content (5.9×10^{-12} g.). The average cell content of RNA was then calculated by dividing the RNA per whole gland by the total number of cells of the gland.

The differences in RNA content per cell do not invariably refer to actual changes in concentration of RNA per unit cell mass because of the marked fluctuations in relative cell sizes as indicated by the average cell height determinations. Furthermore since the

change in total DNA represents a change in number of cells, the change of the ratio

$$\left(\frac{\text{total cell mass}}{\text{total DNA}}\right)$$

versus controls provides a relative index of cell size.

Chemical Methods

All tissues were kept on ice, weighed immediately, and homogenized in chilled Potter-Elvejhem glass homogenizers with chilled 0.25 M. sucrose. If not immediately used for determination of nucleic acids, the homogenate was kept at −20° C.

Aliquots of tissue were dried to constant weight in all glass apparatus with vacuum, P_2O_5, and heating by boiling toluene.

DNA and RNA were isolated by the Schmidt-Thannhauser-Schneider (13) procedure and quantitated in duplicate by the orcinol reaction for ribose of RNA and diphenylamine reaction for desoxyribose of DNA. Yeast RNA and calf thymus DNA were used as standards. The duplicate analyses of DNA agreed within 3%, and of RNA within 4%. The results agreed with those obtained by spectrophotometric estimation of purine and pyrimidine constituents of RNA and DNA. The Schmidt-Thannhauser-Schneider procedure was compared with Schneider's method (14). The RNA data agreed, but the DNA analyses by the Schmidt-Thannhauser-Schneider technique were about 10% lower than found by Schneider's method. Along with analysis of RNA and DNA in the thyroid tissue, both nucleic acids were determined in the rat liver for control purposes. The data on nucleic acid analysis were expressed as μg./mg. wet tissue. The water content of aliquots of tissue was determined so that the results could be related per unit weight of dry tissue. The data on nucleic acid analysis were also expressed per unit of cell mass.

Because the analyses were made from whole tissue homogenates, the contribution to DNA and RNA by the stroma cells was not determinable. However, this was considered relatively constant since the amount of thyroid stroma changed very little in these experiments. In order to determine the nucleic acid content of colloid, sheep thyroid glands were frozen with CO_2. The tissue was cut in slices 10–15μ in thickness, shaken in chilled saline, and centrifuged in the refrigerated centrifuge. Tissue which was resuspended showed retention of follicular structure but was devoid of colloid. The supernatant-containing colloid was used for DNA and RNA determinations. Since no DNA and an insignificant amount (0.13 μg./equivalent 1 mg. wet tissue) of RNA was found in the colloid, the analysis of nucleic acids of the homogenate can be related to cell mass.

RESULTS

The effects of thyrotropin, thyroxine, and thiouracil on the nucleic acids of the guinea pig thyroid gland

Thyrotropin-treated animals. In the thyrotropin-treated animals, the average wet weight of the thyroid increased 128%, while total cell mass of the gland increased 330%. The cell population density was decreased 19%. The average cell height was increased 246%. The DNA per unit weight of wet tissue was slightly increased (10%) while DNA calculated per unit weight of cell mass was markedly decreased (41%). Finally, the total DNA per gland was increased 150%.

The increase in total cell mass along with a decrease in cell population density indicate hypertrophy of thyroid cells, for as cytoplasm volume in-

reased, the cell and nuclear population densities naturally tended to decrease. Furthermore, the hypertrophy of the cell is evident from the proportionally greater increase in total cell mass (330%) than in total DNA per whole gland

$$\left(\frac{\text{increase in total cell mass} = 330\%}{\text{increase in total DNA} = 150\%} = 2.2\right).$$

Therefore this ratio (2.2) is an index of relative cell enlargement. The hypertrophy of the cells is responsible for a great decrease in DNA per unit weight of cell mass.

The inconsistency of an increase in DNA per unit weight of wet tissue coexisting with a decrease in cell population density, apart from a technical error, could be explained by an increased number of cells with polyploid nuclei, i.e., nuclei undergoing mitosis. This phenomenon does not represent a specific effect of thyrotropin on the nuclear content of DNA of each individual thyroid cell, since it has been observed in other growing tissue, such as in the nuclei of the regenerating liver (15). Therefore, these data suggest that the nuclear content of DNA does not change under thyrotropin stimulation.

The RNA per unit weight of wet tissue was considerably elevated (146%), but the increase in RNA per unit of cell mass was less marked (32%). The increase in total RNA per gland was 463%.

TABLE 1. DATA ON CELL HEIGHT, STRUCTURE, CELL POPULATION DENSITY AND I^{131} UPTAKE OF THE THYROID GLAND

	Average cell height, μ	Thyroid cell %	Follicle space %	Stroma %	Cell population density factor = (number of nuclei/ 84,500 μ^2)	I^{131} uptake % of dose
Control	3.7 (0.2)	29.1 (1.8)	50.7	20.1	347 (12)	6.7
Thyrotropin 0.5 U.S.P. sc/day for 14 days	12.8 (0.8)	54.7 (3.5)	24.8	20.5	282 (12)	64.8
	+246%	+87%	−51%	+2%	−19%	+867%
l-sodium thyroxine 100 μg. sc/day for 14 days	3.4 (0.3)	33.8 (2.4)	48.4	17.8	374 (12)	1.0
	−8%	+16%	−5%	−11%	+8%	−85%
Control	5.2 (0.7)	30.4 (1.0)	52.9	16.7	257 (13)	4.8
Thiouracil 0.2% in food for 90 days	12.5 (0.4)	45.5 (0.5)	32.2	22.3	241 (10)	18.5
	+140%	+50%	−39%	+34%	−6%	+285%

Figures in parentheses are standard errors.

The increase in total RNA (463%) over the increase in average weight of the gland (128%) in the thyrotropin-treated animals

$$\left(\frac{\text{increase in total RNA} = 463\%}{\text{increase in gland weight} = 128\%} = 3.6\right)$$

appeared much greater than when correlated with the increase (330%) in total cell mass

$$\left(\frac{\text{increase in total RNA} = 463\%}{\text{increase in total cell mass} = 330\%} = 1.4\right).$$

The latter ratio (1.4) serves as an index of the change of RNA concentration per unit weight of cell mass. The average amount of RNA per cell showed an increase of 125%. The increase in RNA per cell is proportionally greater than the increase of protoplasm in the cell

$$\left(\frac{\text{increase in total RNA} = 463\%}{\text{increase in total cell mass} = 330\%}\right)$$

and therefore the concentration of RNA in the thyroid cell is increased compared to the control gland. These data suggest that RNA is increased in the thyroid cell stimulated by exogenous thyrotropin. The increase in RNA is less marked when related to the unit weight of cell mass than to the unit weight of whole tissue. The increase in total RNA is proportionally greater than the increase in total cell mass.

Thyroxine-injected animals. In the thyroxine-injected animals, the average weight of the thyroid was decreased 32% and the total cell mass of the gland decreased 21%. The average cell height was slightly decreased (8%). The cell population density showed a small increase (8%). The DNA per unit weight of wet tissue was increased (14%) while DNA per unit weight of cell mass was not significantly changed (−2%). The total DNA per gland was decreased 23%. The decrease in total cell mass along with slight increase in cell population density suggest a very small decrease in size of thyroid cells. Furthermore the small decrease in size of

TABLE 2. DATA ON WEIGHT, WATER CONTENT, DNA AND RNA OF THE THYROID GLAND

	Thyroid average weight, mg.	Water %	DNA, μg./mg. wet tissue	DNA, μg./mg. cell mass	RNA, μg./mg. wet tissue	RNA, μg./mg. cell mass	RNA/DNA, μg./wet tissue
Control	47.5	74.0	4.73	16.20	1.96	6.71	0.41
Thyrotropin 0.5 U.S.P. sc/day for 14 days	108.5	78.9	5.18	9.47	4.83	8.83	0.93
	+128%	+7%	+10%	−41%	+146%	+32%	+127%
l-sodium thyroxine 100 μg. sc./day for 14 days	32.2	71.3	5.40	15.93	1.16	3.42	0.22
	−32%	−4%	+14%	−2%	−41%	−49%	−46%
Control	75.9	74.8	4.99	16.42	2.09	6.88	0.42
Thiouracil 0.2% in food for 90 days	314.1	78.0	4.80	10.55	3.22	7.08	0.67
	+314%	+4%	−4%	−36%	+54%	+3%	+60%

thyroid cells is evident from the essentially equal decreases in total cell mass (−21%) and in total DNA per gland

$$\left(\frac{\text{decrease in total cell mass} - 21\%}{\text{decrease in total DNA} - 23\%} = 0.9\right)$$

this ratio (0.9) being an index of relative cell size. The decrease in average weight of the thyroid was more due to decreased water and colloid content of the tissue than to atrophy of the cells. The increase in DNA per unit weight of wet tissue is a relative one and is due to the relative increase in cell population density. This is supported by the fact that there was no significant change in DNA per unit weight of cell mass. These data suggest that the nuclear content of DNA does not change in the absence of thyrotropin stimulation.

The RNA per unit weight of wet tissue was markedly decreased (41%) and the decrease in RNA per unit weight of cell mass was similarly 49%.

The similarity of these two forms of expression merely shows that the proportions of histological constituents did not change compared to the control gland despite a definite decrease in average weight of the gland (32%). The total RNA per gland decreased 60%. The decrease in total RNA per gland (60%) was greater when compared to the decrease (32%) of average weight of the gland

$$\left(\frac{\text{decrease in total RNA} = 60\%}{\text{decrease in gland weight} = 32\%} = 1.9\right)$$

and this ratio was smaller than when the change of RNA was compared with the change (−21%) in total cell mass

$$\left(\frac{\text{decrease in total RNA} = 60\%}{\text{decrease in total cell mass} = 21\%} = 2.9\right).$$

The latter ratio (2.9) is an index of the change of RNA concentration per unit weight of cell mass. The RNA per cell showed a decrease of 48%. The decrease in RNA per cell is proportionally greater than the decrease of protoplasm in the cell

$$\left(\frac{\text{decrease in total RNA} = 60\%}{\text{decrease in total cell mass} = 21\%}\right)$$

and therefore the concentration of RNA in the cell is decreased when compared to the control gland.

These data suggest, that in the absence of thyrotropin stimulation the RNA is decreased in the thyroid cell. The decrease in RNA was very similar whether related to the unit weight of cell mass or to unit weight of wet tissue. The decrease in total RNA per gland was proportionally greater than the decrease in total cell mass.

Thiouracil-fed animals. In the thiouracil-fed animals, the average weight of the thyroid increased 314%, and the total cell mass of the gland increased 519%. The average cell height increased 140%, the cell population density was not significantly changed (−6%). The DNA per unit weight of wet tissue was not significantly changed (−4%), while the DNA per unit weight of cell mass considerably decreased (36%). The total DNA per gland increased 299%. The increase in cell mass with no significant change in cell population density indicate hypertrophy of thyroid cells. The hypertrophy of thyroid cells is evident from a proportionally greater increase in total cell mass than in total DNA per gland

$$\left(\frac{\text{increase in total cell mass} = 519\%}{\text{increase in total DNA} = 299\%} = 1.7\right).$$

The hypertrophy of the cells is responsible for the decrease in DNA per unit weight of cell mass. The DNA per unit weight of wet tissue and the cell population density were in agreement and their decrease was not significant. Therefore, these results suggest that the nuclear content of DNA is not changed when the secretion of endogenous thyrotropin is increased.

The RNA per unit weight of wet tissue was elevated 54%, but the increase in RNA per unit weight of cell mass of the gland was not significant (3%). The total RNA per gland increased 538%.

The increase in total RNA (538%) compared to the (314%) increase in average weight of the gland

$$\left(\frac{\text{increase in total RNA} = 538\%}{\text{increase in gland weight} = 314\%} = 1.7\right)$$

was larger than when correlated with the increase (519%) in total cell mass

$$\left(\frac{\text{increase in total RNA} = 538\%}{\text{increase in total cell mass} = 519\%} = 1.0\right).$$

The latter ratio of approximate unity indicate that the total RNA increase paralleled the increase in the total mass of protoplasm. The average RNA per cell was increased 60%. However, the increase in RNA per cell was proportionally similar to the increase of protoplasm in the cell

$$\left(\frac{\text{increase in total RNA} = 538\%}{\text{increase in total cell mass} = 519\%}\right)$$

and therefore the concentration of RNA in the thyroid cell has not increased.

These data suggest that RNA is increased in the thyroid cell stimulated by increased secretion of thyrotropin. The increase in RNA is not significant when calculated per unit weight of cell mass; for the increase in total RNA per gland is proportionally equal to the increase in total cell mass.

TABLE 3. DATA ON CALCULATED TOTAL THYROID CELL MASS; DNA AND RNA IN WHOLE WET TISSUE, CELL MASS AND IN INDIVIDUAL CELL

	Calculated total thyroid cell mass, (mg.) = (% of cells × aver. gland weight)	DNA/mg. gland $\times 10^{-6}$g.	Total DNA/ gland $\times 10^{-6}$g.	RNA/mg. gland $\times 10^{-6}$g.	RNA/mg. thyroid cell mass $\times 10^{-6}$g.	Total RNA /gland $\times 10^{-6}$g.	Calculated total No. of thyroid cells $\times 10^{6}$	RNA/cell $\times 10^{-12}$g.
Control	13.8	4.73	225	1.96	6.7	93.1	38	2.4
TSH 0.5 U.S.P. sc/day for 14 days.	59.4	5.18	562	4.83	8.8	524	95	5.5
	+330%	+10%	+150%	+146%	+32%	+463%	+150%	+125%
Sodium thyroxine 100 mg. sc/day for 14 days	10.9	5.40	174	1.16	3.4	37.4	30	1.3
	−21%	+14%	−23%	−41%	−49%	−60%	−23%	−48%
Controls	23.1	4.99	379	2.09	6.9	159	64	2.5
Thiouracil 0.2% in food for 90 days.	142.9	4.80	1508	3.22	7.1	1011	256	4.0
	+519%	−4%	+299%	+54%	+3%	+538%	+299%	+60%

The RNA/DNA ratio was increased 127% in thyrotropin-treated and 60% in thiouracil-fed animals, while in thyroxine-injected animals it was decreased 46%. The same changes in RNA/DNA ratio are obtained if RNA and DNA are expressed per unit of cell mass.

The uptake of I^{131} by the thyroids was greatly increased in thyrotropin-treated and very decreased in the thyroxine-injected animals. The increased uptake in thiouracil-fed animals was due to increased trapping of inorganic I^{131}, without formation of significant amounts of protein-bound I^{131} (0.2%), and by chromatography of trypsin-digested thyroid homogenate practically all I^{131} was found to be iodide.

DISCUSSION

The data indicate that histological structure should be considered in the evaluation of chemical assays on a non-solid organ, such as the thyroid gland. When expressed in the conventional manner per unit wet weight of thyroid tissue or per whole gland, chemical analyses may be misleading. For example, the cell mass in the experimental thyroids of this study ranged from a low of 29.1% (control) to a high of 54.7% (thyrotropin-treated) so that a direct comparison of chemical assays on one mgm. samples of these two glands would be made upon unequal samples of protoplasm. This is also evident from the differences of percentage changes of RNA as expressed per unit weight of whole tissue, per unit weight of cell mass and per whole gland and illustrates how comparisons are actually made on different volumes of protoplasm.

These results suggest that thyrotropin does not affect the nuclear content of DNA. The change in DNA per unit weight of wet tissue or per whole gland under the stimulation of increased amount of exogenous or endogenous thyrotropin reflects an absolute increase in the number of cells. The increase in concentration of DNA in the absence of thyrotropin (in thyroxine-injected animals) corresponds with the relative increase in

number of cells due to shrinkage of follicles and stroma, while the decreased DNA content per whole gland suggests a decrease in total number of cells. The greater increase in DNA per whole gland of thiouracil-fed animals than in thyrotropin-treated animals (229%:150%) most probably means that hyperplasia of thyroid cells is greater in the former than in the latter group. The polyploidy of the nuclei is not significantly responsible for the increase of total DNA in thiouracil-fed animals because Santler (16) observed six mitoses/300,000 cells in control, and only one mitosis/800,000 cells in thyroid glands of rats fed thiouracil for 30 days. Since in this experiment animals were fed thiouracil for 90 days, probably a relatively small number of cells was undergoing mitosis at the time of sacrifice. On the other hand, the greater hypertrophy of cells in the thyrotropin-treated group than in the thiouracil-fed animals is corroborated by a correlation of change in total cell mass of the gland with the change in total DNA per gland,

$$\left(\frac{\text{increase in total cell mass} = 330\%}{\text{increase in total DNA} = 150\%} = 2.2\right)$$

in the thyrotropin-treated, and

$$\left(\frac{\text{increase in total cell mass} = 519\%}{\text{increase in total DNA} = 299\%} = 1.7\right)$$

in the thiouracil-fed animals. Therefore, these data suggest that during the short but very intensive stimulation of the thyroid by thyrotropin, the hypertrophy of cells contributed relatively more than hyperplasia to the enlargement of the gland.

These data also suggest that the stimulation of the thyroid with increased amounts of exogenous and endogenous thyrotropin is intimately associated with an increase of total RNA per gland. This is also evident by a great decrease in RNA in the absence of thyrotropin stimulation as observed in thyroxine-injected animals.

The effect of thyrotropin on RNA is much less pronounced if the RNA is related to the unit weight of cell mass of the gland rather than to the unit weight of whole, wet tissue. The data also show that the increase in RNA was smaller with prolonged stimulation by endogenous thyrotropin than in short-term and intensive stimulation with exogenous thyrotropin. The smaller increase in the RNA of thiouracil-fed than of thyrotropin-treated animals could conceivably be due to an inhibitory effect of thiouracil *per se* on the synthesis of RNA, as has been observed in tobacco mosaic virus (17) and bacterium coli (18). However, since RNA is related to protein synthesis and growth of the cells, it is difficult to reconcile this explanation with the fact that the increase in average weight of the thyroid was greater in thiouracil than in thyrotropin-treated animals. Therefore, it is probable

that thiouracil *per se* does not significantly inhibit the synthesis of RNA in the thyroid gland.

The present observations of: 1. a relatively constant DNA content of individual nuclei; 2. increases or decreases of RNA in activated or inactivated thyroid cells respectively were not reported in previous studies on nucleic acids in the thyroid gland by Dempsey and Singer (1); Reich (2); Roels (4); and Rerabek and Rerabek (5); and Van Erkelns (7). The results of present studies are essentially in agreement with the observations of Suzuki (6); Alfert, Bern and Kahn (3); and Fiala, Sproul and Fiala (8).

Close quantitative comparisons of the results of previous and present studies are not possible because different laboratory techniques were used and in none of the previous studies were correlations made between the chemical change of nucleic acids and the change of the structure of the thyroid gland.

Acknowledgments

The authors express gratitude to Dr. J. B. Stanbury for advice and assistance and to other members of the Thyroid Clinic for aid. Thanks are due to Drs. J. F. Scott and L. Hecht for advice on chemical methods and to Dr. E. B. Taft for advice on the histological methods.

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LIMITATIONS OF THE CHICK RADIOPHOSPHORUS UPTAKE ASSAY AND THE CHICK RADIOIODINE DEPLETION ASSAY FOR THYROTROPIC HORMONE WHEN APPLIED TO BLOOD AND URINE[1,2]

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ABSTRACT

The chick thyroid P^{32} uptake and I^{131} depletion methods for the bioassay of anterior pituitary thyrotropic hormone (TSH) have been applied to the measurement of TSH in human serum and urine. Although there are detectable levels of TSH in sera from some patients with myxedema, hyperthyroidism, or exophthalmos, the levels in normal individuals are too low to measure accurately. The ultrafiltrate residue of urine contains a non-hypophyseal factor which stimulates the uptake of P^{32} by the chick thyroid, which imposes limitations on the application of the P^{32} uptake assay to urine. Urine ultrafiltrate residue also contains a non-hypophyseal factor which interferes with the effect of TSH on I^{131} depletion in the chick thyroid. Assay of TSH in human serum or urine by these methods will require, therefore, either concentration of TSH from serum or elimination of interfering factors from urine.

THE uptake of radiophosphorus (P^{32}) by the thyroid gland of the chick, and the depletion of radioiodine (I^{131}) from the chick thyroid have been developed into simple and sensitive procedures for the bioassay of anterior pituitary thyrotropic hormone (TSH) (1, 2, 3, 4). These methods are adaptable to a 4- or 6-point bioassay design (5) which may then be applied to the measurement of TSH in human serum and urine. Substances interfering with the bioassay of TSH have been found in serum by Postel (6) and in urine by Greenspan *et al.* (7) and Starr *et al.* (8). This report deals with the limitations of the chick P^{32} uptake assay and the chick I^{131} depletion assay, as applied to blood and urine, because of the presence of nonspecific reactive substances and interfering substances.

MATERIALS AND METHODS[3]

Blood: Fresh or frozen sera, or blood specimens prepared by the method of de Robertis (9), were examined.

[1] This work was supported in part by Grant Number A-568 from the National Institute of Arthritis and Metabolic Diseases, Public Health Service.

[2] Presented to the American Goiter Association June 18, 1958.

[3] TSH standard preparation Lot No. 6, used in these experiments, was kindly supplied by Armour Laboratories, Kankakee, Illinois.

Urine: Twenty-four hour specimens of urine, collected under refrigeration, were ultrafiltered through a 2% collodion membrane according to the method of Gorbman (10). The membrane was removed with anhydrous alcohol-ether and the dried ultrafiltrate residue (UFR) was dissolved in saline prior to assay. In the presence of a small amount of carrier protein, quantitative recovery of purified TSH was achieved by ultrafiltration. The dose of urine UFR is expressed in "hours," *i.e.*, one hour of urine UFR represents 1/24 of a daily urine collection. Twenty-four hours of human urine UFR contained about 50 mg. of protein, as well as small amounts of Na, K, Mg, and Ca. Doses of 4 hours of human urine UFR produced no evident toxic effects in recipient chicks.

The P^{32} uptake assay: The details of this assay, based upon the uptake of P^{32} by the chick thyroid 6 hours after intracardiac administration of TSH or test substance, have been reported (1). The response is expressed as $\sqrt{t}$, where t equals the time required to count 1024 counts per thyroid. Increased P^{32} uptake induced by TSH is indicated by decreased $\sqrt{t}$. The useful range of the assay is 0.25 to 5 milliunits of TSH (1 milliunit, abbreviated "mu." equals 0.001 U.S.P. unit of TSH). The precision of a series of assays, using groups of 5 chicks per dose, was indicated by a mean lambda of 0.34.

The I^{131} depletion assay: The method of Bates and Cornfield (4), based upon the depletion of I^{131} from the thyroid glands of chicks treated with thryoxine and propylthiouracil, was found satisfactory only with chicks having a high spontaneous uptake of I^{131}.[4] The response is expressed as per cent of the initial content of I^{131} remaining 24 hours after subcutaneous administration of test substances. Increased TSH potency is indicated by decreased per cent I^{131} remaining. The useful range of the assay was 1 to 16 mu. TSH, and with groups of 5 chicks per dose the mean lambda for a series of assays was 0.22.

Other methods of study: Twenty-four hour I^{131} uptakes were determined in thyroxine treated chicks after 2 days of administration of test substances. Histological examination of chick thyroids was made after 3 days of administration of test substances. These techniques were comparatively insensitive to TSH but were useful to demonstrate substances interfering with TSH.

RESULTS

Attempts to assay TSH in serum

Limitations of the P^{32} uptake assay: We were unable to detect TSH activity in serum from normal subjects, patients with Graves' disease, or patients with malignant exophthalmos. TSH was not detectable in blood processed by the method of de Robertis (9) nor in serum protein fractions prepared by continuous flow paper electrophoresis according to the method of Durrum (11) (Table 1). Although the P^{32} uptake assay will detect 0.25 mu. of TSH, intracardiac injection of test substances limits the injection volume to 0.1 cc., so that the minimal detectable amount of TSH in unconcentrated serum would be 2.5 mu. per ml. Application of this assay technique to untreated serum is limited to specimens containing very high levels of TSH.

Limitations of the I^{131} depletion assay: This assay will usually detect 1 mu. of TSH; and since a maximum of 2 ml. of serum can be administered

[4] White leghorn cockerels, obtained from Kimber Hatchery, Niles, California, were satisfactory until June 1958, when I^{131} uptake diminished due to addition of iodide to the diet.

TABLE 1. THE EFFECT OF HUMAN SERUM AND BLOOD FRACTIONS ON P^{32} UPTAKE OF CHICK THYROID

Source of serum or blood	Dose,† ml./chick	P^{32} uptake response*			
		Serum or blood $\sqrt{t}+2$ SE	Control $\sqrt{t}+2$ SE	TSH standard	
				Dose, mu.‡	Response, $\sqrt{t}+2$ SE
Normal serum	0.1	11.94 ± 0.82	12.27 ± 1.21	0.5	10.15 ± 1.13
Exophthalmos serum	0.1	10.80 ± 0.32	10.61 ± 1.19	0.75	7.89 ± 0.67
Hyperthyroid serum	0.1	12.59 ± 0.63	12.30 ± 1.05	0.5	10.93 ± 0.47
Normal blood,§ 85% acetone precipitate	0.5	10.15 ± 1.00	11.57 ± 1.30	2.0	7.29 ± 0.82
Normal serum:‖					
Albumin	0.13	10.01 ± 0.20	11.07 ± 1.28	0.5	11.00 ± 1.21
$\alpha+\beta$ Globulin	0.13	10.92 ± 0.60		1.5	8.67 ± 1.02
γ Globulin	0.17	11.21 ± 1.00		4.5	6.90 ± 1.11

* Response expressed as $\sqrt{t}$, where t equals time to count 1024 cts. per thyroid. Values represent mean ±2 standard errors for groups of 5 chicks.

† Dose of fresh serum, or the amount of blood or serum represented by 0.1 ml. of concentrate.

‡ 1 mu. TSH equals 0.001 U.S.P. unit of TSH.

§ Blood processed by method of de Robertis (9).

‖ 2.5 ml. serum fractionated by continuous flow paper electrophoresis method of Durrum (11).

to the chick subcutaneously, the minimal detectable concentration of TSH in serum would be 0.5 mu. per ml. The concentration of TSH in serum would have to be considerably higher than this to permit application of a 4-point bioassay procedure. Table 2 shows the results of a series of assays in which the I^{131} depletion response of 2-ml. portions of serum from vari-

TABLE 2. ACTIVITY OF SERA FROM VARIOUS PATIENTS IN THE I^{131} DEPLETION ASSAY

Diagnosis	Patient		I^{131} depletion response*			
	Sex	Age	Control, % R ±2 SE*	TSH, 1 mu. % R ±2 SE†	Serum, 2 ml. % R ±2 SE	P‡ value
Normal	F	36	86.4 ± 3.0	66.5 ± 5.8	82.8 ± 2.8	> .05
	F	31	79.5 ± 3.2	78.5 ± 3.4	80.9 ± 5.0	> .05
	F	44	88.8 ± 4.0		78.6 ± 7.0	> .02, < .05
	M	38	84.6 ± 2.4	72.3 ± 8.2	72.9 ± 11.0	.05
	M	30	92.2 ± 7.0	77.6 ± 8.2	83.3 ± 1.5	.05
Myxedema	F	50	84.6 ± 2.4	72.3 ± 8.2	76.7 ± 7.4	.05
	F	55	100.8 ± 6.4	92.4 ± 6.4	80.4 ± 5.2	.01
	F	50	85.6 ± 3.2	76.5 ± 11.4	75.0 ± 11.4	> .05
	F	52	86.5 ± 4.2	75.4 ± 5.8	74.2 ± 11.2§	> .05
Graves' Disease	F	38	100.8 ± 6.4	92.4 ± 6.4	90.4 ± 6.8	> .05
	F	55	100.8 ± 6.4	92.4 ± 6.4	84.8 ± 5.6	< .01
Malignant Exophthalmos	M	50	92.0 ± 3.4	84.1 ± 2.6	76.7 ± 1.5	< .01
	M	30	74.7 ± 7.8	77.6 ± 12.4	67.7 ± 11.5	> .05
Pituitary Myxedema	F	71	85.6 ± 3.2	76.5 ± 11.2	84.6 ± 3.8	> .05

* Response (%R) expressed as % initial thyroid content of I^{131} remaining 24 hours after test substance, mean ±2 standard errors for groups of 5 chicks.

† This is a single point from the TSH dose response curve presented to indicate the sensitivity of the assay. 1 mu. TSH equals 0.001 U.S.P. unit of TSH.

‡ P value for comparison of serum group with control group.

§ Response to 0.6 ml. serum.

ous patients was compared with a TSH standard curve run simultaneously. Sera from normal subjects revealed barely significant I^{131} depletion activity. Although it is not obvious from the individual assays, it can be shown by pooling the data that sera from patients with myxedema, Graves' disease, and malignant exophthalmos were more active, but the activity was still not high enough to permit utilization of a 4-point bioassay procedure. Thus, even with the increased precision achieved by use of larger groups of animals, it is apparent that the I^{131} depletion assay is also limited to sera containing high levels of TSH.

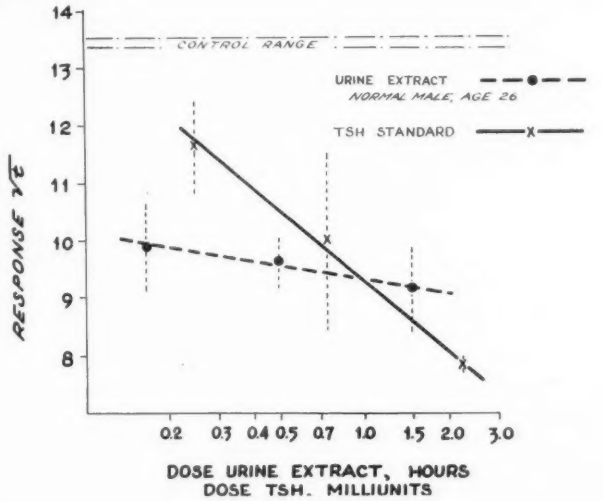

FIG. 1. Effect of TSH and urine UFR on P^{32} uptake assay. Each point represents mean response ± 1 standard deviation for group of 5 chicks. In this and in succeeding charts, the dose of urine UFR is expressed in hours, where 1 hour equals 1/24 of a daily urine collection.

Attempts to assay TSH in urine ultrafiltrate residue

Limitations of the P^{32} uptake assay: We have found that urine UFR from all subjects tested stimulated the uptake of P^{32} by the chick thyroid. Specimens of urine UFR from normal individuals were quite active in the P^{32} uptake assay, but the dose-response curves were flat (Fig. 1). In 60 assays of urine UFR from various patients, the slopes of the urine curves were significantly less than the slopes of the standard TSH curves. This suggested that the factor in urine UFR active in the P^{32} uptake assay was different from TSH or that some interfering factor was present. Specimens of urine UFR from 5 patients with clinical and laboratory evidence of panhypopituitarism were found to have considerable activity in this assay (Table 3). In these subjects, we did not have autopsy proof of the completeness of hypophysectomy. However, we have assayed samples of

TABLE 3. EFFECT OF URINE UFR FROM PATIENTS WITH PANHYPOPITUITARISM ON THE P^{32} UPTAKE OF CHICK THYROIDS

Patient	Clinical diagnosis*	Control response, $\sqrt{t} \pm 2$ SE†	TSH dose,‡ mu.	TSH response, $\sqrt{t} \pm 2$ SE†	UFR dose, "hrs."	UFR response, $\sqrt{t} \pm 2$ SE†
Don	Craniopharyngioma	15.23 ± 0.81	0.75	11.49 ± 1.24	3.0	11.08 ± 1.34
Tak	Craniopharyngioma	10.41 ± 0.66	0.75	9.13 ± 0.71	1.5	8.33 ± 0.68
McC	Chromophobe adenoma	13.61 ± 1.91	0.75	10.73 ± 1.21	1.5	8.95 ± 0.61
Bas	Hypophysectomy§	13.71 ± 0.75	0.75	9.67 ± 1.19	1.5	7.86 ± 1.13
Zwy	Hypophysectomy§	13.71 ± 0.75	0.75	9.67 ± 1.19	1.5	9.67 ± 1.27

* All patients had clinical and laboratory evidence of thyroid insufficiency with panhypopituitarism.

† Response expressed as $\sqrt{t}$, where t equals time required to count 1024 cts. per thyroid, ± 2 standard errors. Groups of 5 chicks per dose.

‡ This is a single point from the TSH dose response curve presented to indicate the sensitivity of the assay.

§ Patients with advanced breast carcinoma hypophysectomized by irradiation with deuteron beam. Specimens were obtained through the kindness of Dr. J. N. Lawrence, Donner Laboratory, University of California, Berkeley, California.

urine UFR from normal and totally hypophysectomized dogs[5] and have found considerable activity in each. The dose-response curves from such an assay are presented in Figure 2. Urine UFR from both the normal and the hypophysectomized dog contained essentially the same activity in the P^{32} uptake assay. Within the range tested, there was no increase in response with increased dosage. This effect is considerably different from that of TSH. These findings suggest that there is in urine UFR a non-hypophyseal factor which stimulates the uptake of P^{32} by the chick thyroid. This greatly interferes with the application of the P^{32} uptake assay to the study of TSH in urine.

Limitations of the I^{131} uptake assay: Urine UFR from normal subjects was consistently active in the I^{131} depletion assay, whereas urine UFR from patients with panhypopituitarism was inactive. However, the dose response curve of human urine UFR in this assay was invariably flatter than the TSH curve (Fig. 3). Furthermore, when urine UFR was administered to the chick in a separate site but simultaneously with TSH, the resultant I^{131} depletion dose response curve was significantly modified (Fig. 3). These findings indicated the presence of a factor in normal human urine UFR which interfered with the effect of TSH upon the discharge of I^{131} from the chick thyroid gland. Urine UFR from normal and hypophysectomized dogs was similarly studied. Urine UFR from normal dogs was usually active in the I^{131} depletion assay although the dose-response curve was flat. Urine UFR obtained from hypophysectomized dogs was consistently inactive. This confirmed our observations on human urine UFR, and also implies that the I^{131} depletion response is probably more specific for TSH than the P^{32} uptake response. When urine UFR from normal or hypophysectomized dogs was administered to the chick in separate sites

[5] These specimens were kindly supplied by Dr. W. F. Ganong, Department of Physiology, University of California School of Medicine.

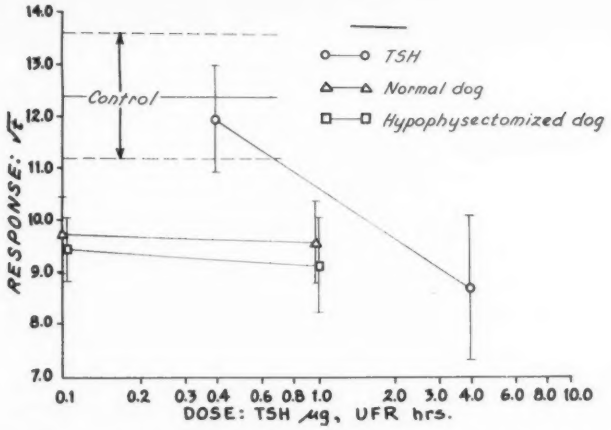

FIG. 2. Effect of urine UFR from hypophysectomized and normal dogs in P^{32} uptake assay. Each point represents mean ±1 standard deviation for group of 5 chicks.

but simultaneously with standard TSH, the expected TSH-induced I^{131} depletion response was partially inhibited (Fig. 4). Although in this experiment there was no spontaneous TSH-like activity in the urine UFR from either the normal or the hypophysectomized animal, 2 hours of urine UFR clearly interfered with the activity of 4 mu. of TSH. A similar type of interference with TSH action was obtained when I^{131} uptake was studied. In chicks with a low spontaneous I^{131} uptake,[6] administration of 50 mu.

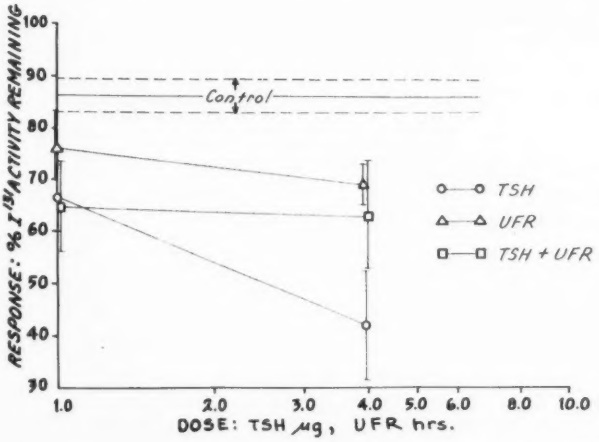

FIG. 3. Separate and combined effects of normal human urine UFR and TSH in I^{131} depletion assay. Each point represents mean ±1 standard deviation for group of 5 chicks.

[6] White Leghorn cockerels with low I^{131} uptakes were obtained from Poehlmann Hatcheries, Petaluma, California.

of TSH induced a significant increase in uptake. This effect of TSH was completely blocked by the simultaneous administration of 2 hours of urine UFR from normal or hypophysectomized dogs (Fig. 5). Comparable effects were obtained with urine UFR from normal humans.

Urine UFR from human subjects was also able to block the effect of TSH on the histological structure of the chick thyroid. In this experiment, groups of 6 chicks received daily injections of 20 mu. of TSH, or 2 "hours" of human urine UFR, or both, for three days. Twenty-four hours after the last injection the thyroids were removed for histological examination.

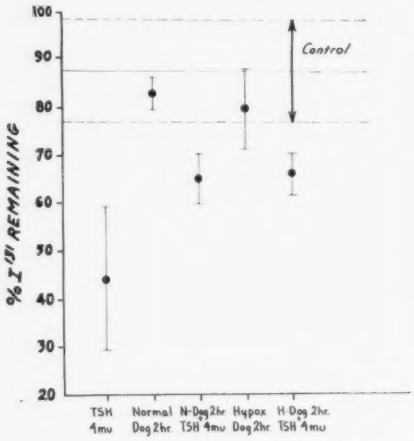

FIG. 4. Effect of urine UFR from normal and hypophysectomized dogs in the I^{131} depletion assay. Each point represents mean ± 1 standard deviation for a group of 5 chicks.

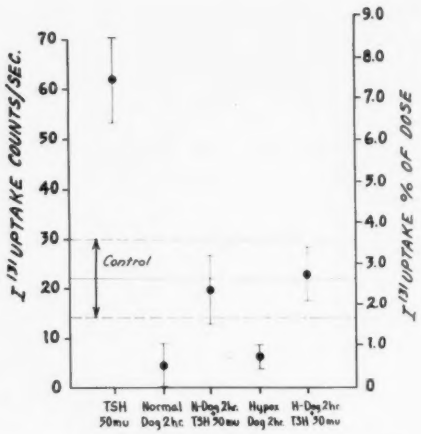

FIG. 5. Effect of urine UFR from normal and hypophysectomized dogs on the I^{131} uptake of chick thyroids. Each point represents mean ± 1 standard deviation for a group of 6 chicks.

Representative sections are presented in Figure 6. TSH induced marked cellular hyperplasia and loss of colloid. This was completely blocked by the simultaneous administration of urine UFR.

These data clearly indicate that there is in human and animal urine a factor which interferes with the action of TSH on the chick thyroid gland. Since it is present in urine from hypophysectomized animals, it is not of hypophyseal origin. The presence of this interfering factor limits the application of the I^{131} depletion assay to urine.

Attempts to separate TSH from interfering substances in human urine UFR

Extensive studies have been made in an effort to separate TSH from interfering substances in urine UFR. No separation of these two activities could be made by treatment of urine UFR with heat, pepsin hydrolysis, sodium periodate, acid hydrolysis, 10% trichloracetic acid extraction,

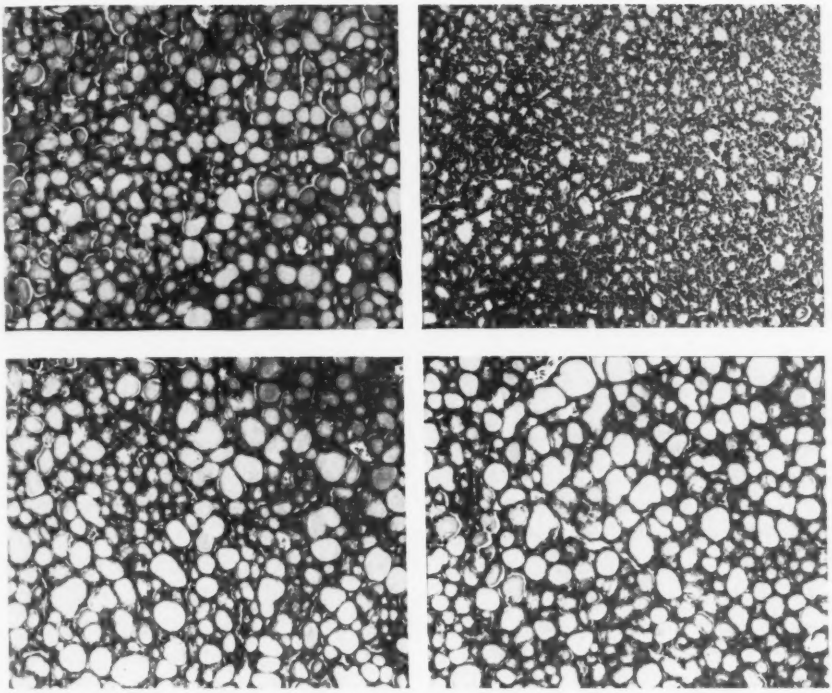

FIG. 6. Effect of TSH and human urine UFR on the histological structure of the chick thyroid. (Hematoxylin and eosin stained sections magnified 220×.) 1. (Upper left) Control 2. (Upper right) 0.02 U.S.P. units of TSH daily for 3 days. 3. (Lower left) 2 hours of UFR daily for 3 days. 4. (Lower right) 0.02 U.S.P. units of TSH plus 2 hours of UFR daily for 3 days.

acid acetone or alkaline acetone fractionation, or separation between n-butanol and p-toluenesulfonic acid (12). We have recently applied the method of Bates and Condliffe (13), using decreasing concentrations of ethanol saturated with NaCl, to elute a mixture of urine UFR and filter aid (Hyflo Super-cel[7]). Preliminary data indicate that bovine TSH may be recovered at a concentration of 65% to 70% ethanol saturated with NaCl. Urine UFR also yields an active fraction eluted at 70% ethanol-NaCl. Further studies are in progress to characterize the relative concentrations TSH and TSH interfering substances in these fractions.

DISCUSSION

Our data suggest that the levels of TSH in normal sera are below 0.5 mu. per ml., confirming the reports of Gilliland Strudwick (3), DiGeorge *et al.* (14), and McKenzie (15). It is possible that high serum TSH levels in patients with myxedema and exophthalmos can be determined by the

[7] Kindly supplied by Johns-Manville Company, New York 16, New York.

I^{131} depletion assay in a 4-point system, but the levels found in normal individuals are too low to measure by this method. We have evidence that large amounts of inactive serum protein will flatten the dose response curve of purified TSH in both the P^{32} uptake assay and the I^{131} depletion assay. In addition, larger amounts of serum will probably reveal evidence of the factor interfering with TSH described by Postel (6). Isolation of TSH from serum may be difficult since Querido has reported that 2 sera fractionated by the Cohn technique yielded TSH activity in different fractions (16). Further study of serum fractionation is indicated, as is exploration of more sensitive methods of TSH bioassay, such as I^{131} discharge in the mouse as reported by McKenzie (15) or I^{131} uptake of tissue culture preparations of guinea pig thyroid recently described by Bottari (17).

The presence of a non-hypophyseal factor in human and dog urine UFR which is active in the P^{32} uptake assay is quite surprising. The stimulation of the uptake of P^{32} in chick thyroid has been considered a specific effect of TSH (1, 2). Indeed, the only substance other than TSH and urine UFR active in this assay has been cobalt (18). Studies on the distribution of P^{32} in various phosphate-containing fractions of chick thyroid tissue processed by the method of Schneider (19) have revealed differences in the effects of these 3 substances. In these studies, chick thyroid glands were fractionated into acid soluble phosphorus (ASP), lipid soluble phosphorus (LSP), nucleic acid phosphorus (NAP), and phosphoprotein (PP). TSH markedly increased P^{32} uptake into all of these fractions. Cobalt induced a marked rise in P^{32} content of the ASP fraction with slight depletion of P^{32} from the other fractions. Human urine UFR, like TSH, induced significant increments of P^{32} into all fractions, but there was a somewhat greater rise in the P^{32} content of the ASP fraction, and a lesser rise in P^{32} content of NAP. Thus the effects of urine UFR were intermediate between those of cobalt and TSH. The nature of the factor in urine UFR inducing this reaction will require further study. The presence of this factor in urine UFR presents, however, a definite limitation in the use of the P^{32} uptake assay as applied to urine.

The presence of a factor in urine which interferes with the action of TSH is of great importance in evaluating human TSH bioassay data. Since this factor is present in the urine of hypophysectomized dogs, it is not an inactivated or acetylated TSH with blocking effect, as described by Sonenberg and Money (20). It is present in urine extracts prepared by acetone precipitation (9), indicating that it is not an artifact induced by ultrafiltration. Data on urine levels of TSH reported by Bloch-Michel (21) and Currie (22) may have to be corrected for the presence of an interfering substance. This factor interfering with TSH is also of considerable theoretical interest. Although it may represent some non-specific toxic effect of serum or urine, it could represent an extrapituitary mechanism for modifying thyroid function. If it can be separated from TSH in serum

or urine, we shall be able to determine more precisely its role in thyroid physiology.

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THE ENDOCRINE SOCIETY

1959 Annual Meeting

The Forty-First Annual Meeting of The Endocrine Society will be held in the Haddon Hall Hotel, Atlantic City, New Jersey, Thursday, Friday, and Saturday, June 4, 5, 6, 1959.

The Chairman of Local Arrangements is Dr. Matthew Molitch.

Scientific Sessions will be held from 9:00 A.M. to 5:00 P.M. daily, and in addition there will be simultaneous afternoon sessions. The annual dinner is scheduled for Friday, June 5th at 7:30 P.M. preceded by cocktails at 6:30 P.M.

All members are urged to make their hotel reservations immediately. The Chalfonte-Haddon Hall Hotel will hold 300 bedrooms for members until May 1, 1959, after which time the hotel will not guarantee further reservations. Therefore it is imperative that you make your reservations now directly with the hotel, advising them of time and date of arrival and departure. Make your reservations now and avoid disappointment.

Final program, membership card and advance registration forms will be sent on May 1st 1959 to members whose current dues have been paid.

Those wishing to present papers, which will be strictly limited to ten minutes, should send four copies of the title and abstract to the Vice-President, Dr. Frank Engel, Duke Hospital, Durham, North Carolina, not later than February 1, 1959. It is imperative that the abstracts be informative and complete with results and conclusions—not a statement that those will be presented at the meeting—in order that they may be of reference value and suitable for printing in the program. The reading and processing of approximately two hundred abstracts submitted each year and compiling of the program from these involves a tremendous amount of time and effort on the part of the Program Committee. The Council requests that authors adhere strictly to the following rules when submitting abstracts, otherwise they cannot be considered:

1. IT IS ASSUMED THAT ABSTRACTS SUBMITTED FOR THIS PROGRAM HAVE NOT AND WILL NOT BE SUBMITTED ELSEWHERE.

2. Abstracts may not exceed two hundred words, or equivalent space, exclusive of title. No footnotes or acknowledgements to sponsors can be published. Reference, if used, must be placed in the body of the text. The abstract should consist of a single paragraph, if possible. Structural chemical formula cannot be used.

3. The title heading must be arranged as follows:
   Line 1. Title, not to exceed fifteen words.

Line 2. Author/s. The name of each non-member author collaborating with member-authors is to be followed by the phrase "(by invitation)." Names of non-members who are introduced, i.e., who are not collaborators with member-authors, are to be followed by the phrase "(introduced by . . .)." The principal degree, e.g., M.D., of each author should be written after his name.

Line 3. Institution of origin and city in which institution is located.

4. The body of the abstract, typed double space, should follow the heading. The original copy should be on bond paper. There should be three copies.

5. Abstracts should be letter perfect, since there will be no opportunity for proof reading by the authors.

1959 AWARDS

The selection of the recipients of the awards of The Endocrine Society is made by a Committee appointed by the Council of the Society. These awards and fellowships carry no obligation by the recipient to the Society or to the donors.

Fred Conrad Koch Award

During the past year a substantial legacy has been bequeathed the Society by the late Elizabeth Koch for the purpose of establishing the Fred Conrad Koch Memorial Fund in memory of her late husband, distinguished service professor of physiological chemistry at the University of Chicago and pioneer in the isolation of the androgens. This is to be the highest honor of the Endocrine Society and is to be represented by a medal that is to be known as the Koch Medal of The Endocrine Society. The medal and honorarium of $3,500 is to be given annually to an individual for work of special distinction in endocrinology.

The recipient shall be chosen from nominations presented by members of the Society and is limited to citizens of the United States and Canada. This award will replace the Medal of the Endocrine Society which was established in 1954 and presented to Dr. Carl Moore in 1955, Dr. Frederick L. Hisaw in 1956, Dr. Joseph C. Aub in 1957 and Dr. I. L. Chaikoff in 1958. The Endocrine Medal replaced the E. R. Squibb Award which was formerly the highest honor bestowed by the Society. Past recipients of the Squibb Award were Dr. George W. Corner in 1940, Dr. Philip E. Smith in 1941, Dr. Fred C. Koch, 1942, no award was given in 1943, Dr. E. A. Doisy in 1944, Dr. E. C. Kendall in 1945, Dr. Carl O. Hartman in 1946, Drs. Carl F. and Gerty T. Cori in 1947, Dr. Fuller Albright in 1948, Dr. Herbert Evans in 1949, Dr. C. N. H. Long in 1950, Dr. J. B. Collip in 1951, Dr. James H. Means in 1952 and Dr. David Marine in 1953.

The Ciba Award

The Ciba Award, to recognize the meritorious accomplishments of an investigator not more than 35 years of age in the field of clinical or preclinical endocrinology, was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B Astwood, 1945 Dr. Jane A. Russell, 1946 Dr. Martin M. Hoffman, 1947 Dr. Choh Hao Li, 1948 Dr. Carl Heller, 1949 Dr. George Sayers, 1950 Dr. Oscar M. Hechter, 1951 Dr. Albert Segaloff, 1952 Dr. Seymour Lieberman, 1953 Dr. Sidney Roberts and Dr. Clara Szego (Mrs Roberts), 1954 Dr. Isadore M. Rosenberg, 1955 Dr. Jack Gross, 1956 Dr. Alfred M. Bongiovanni, 1957 Dr. Nicholas S. Halmi, 1958 Dr. Monte Arnold Greer. Prior to 1952 the Award was $1,200. It has now been increased to $2,500.

The Ayerst and the Squibb Fellowships

The Ayerst Fellowship was established in 1947 and the Squibb Fellowship in 1956. They are designed to assist men or women of exceptional promise in furthering their advancement towards a career in endocrinology. Each Fellowship is awarded on alternate years and the stipend, which will not exceed $5,000 may be divided into two Fellowships in varying amounts in accordance with the qualifications of the appointees. Individuals possessing the M.D. or Ph.D. degree, or candidates for either of these degrees, are eligible for appointment.

Applicants must submit the following information:

1. Evidence of scientific ability as attested by studies completed or in progress.
2. Recommendations from individuals familiar with the candidate and his work.
3. A proposed program of study.
4. Acceptance of the individual by the head of the department in which the Fellowship will be held.
5. A statement that he or she will serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be spent in course work or participation in teaching, the latter purely on a voluntary basis.

The Schering and the Upjohn Scholars of The Endocrine Society

The Council of The Endocrine Society has established a category of Scholars. These grants have been made available through the generosity of the Schering Corporation and the Upjohn Company, and will be awarded to established investigators and teachers in the field of endocrinology who wish to extend their opportunities for work either in this country or abroad.

The awards will not exceed $2,500 annually for each individual and will be granted on the basis of proposals submitted by the applicant. Such applications should include estimated financial needs. The funds may be used for travel, maintenance and other expenses.

Nominations

Nominations for the Fred Conrad Koch Award; the Ciba Award; and the Ayerst and the Squibb Fellowships may be made by any member of The Endocrine Society. They should be submitted on forms which may be obtained from the Office of the Secretary, 1200 North Walker Street, Oklahoma City 3, Oklahoma. Completed nominations should be returned to the Secretary not later than October 15, 1958.

Proposals for appointments as Scholars of The Endocrine Society should be made in writing by the individual, and addressed to the Secretary of the Society. They should be submitted by October 15, 1958.

The Awards Committee will meet in October and notice of awards to successful nominees and applicants will be made not later than December 1st.